BEAN YELLOW MOSAIC AND CLOVER YELLOW VEIN VIRUSES: PURIFICATION, CHARACTERIZATION, DETECTION AND ANTIGENIC RELATIONSHIPS OF THEIR NUCLEAR INCLUSION PROTEINS

BY

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Bean yellow mosaic virus (BYMV) and clover yellow vein virus (CYVV) induce proteinaceous cylindrical inclusions (CI) and nuclear inclusion (NI) in infected tissues. The purpose of this study was to isolate the NI and to characterize the proteins associated with them.

The NI induced by BYMV and CYVV in pea were purified by treating the tissue extracts with Triton X-100 followed by low speed centrifugation. The NI of both viruses contained two distinct species of protein monomers. The molecular weights (Mr.) of the BYMV NI monomers were 54k and 49k, while those of the CYVV monomers were 60k and 49k. The two NI protein monomers of BYMV and CYVV were virus-specified and were antigenically and chemically distinct from each other and from CI and capsid proteins. A 98k protein and a 100k protein were consistently associated with NI preparations of BYMV and CYVV, respectively. These high Mr. proteins were shown to be

polyproteins which contained sequences from both large and small NI monomers.

Based on immunoprecipitation analysis of in vitro translation products stimulated by BYMV RNA in the rabbit reticulocyte lysate (RRL) and wheat germ lysate systems, the genome map for BYMV from the 5'-terminus to the 3'-terminus is proposed as follows: 32k--unknown protein, 48k--HC, 30k--unknown protein, 73k--CI, 12k--unknown protein, 49k--NI, 54k--NI and 32k--CP.

With deletion of dithiothreitol (DTT) from the RRL system, products with Mr. greater than 200k were detected. These products, however, were readily converted to lower Mr. products once DTT was added, indicating that the proteolytical processing of polyproteins was the translation strategy of BYMV RNA. This proteolytical processing activity was inhibited by treating the lysate with antiserum to 49k NI, indicating that this protein or a related protein is a virus-specified protease.

The NI proteins were detected in extracts of virus infected tissues by SDS-immunodiffusion, indirect ELISA, and Western blotting tests and in situ by immunofluorescence tests. Using immunofluorescence tests, the plasmalemma was found to be a possible site for the synthesis of CI, CP, small NI and large NI proteins of BYMV as early as 48 hours after inoculation.

Among strains of BYMV, the 54k NI protein was the most antigenically conserved of the four BYMV specified proteins examined. Strains of BYMV were readily distinguished from CYVV and ten other potyviruses based on antigenic properties of the BYMV 54k NI protein.

CHAPTER 1
INTRODUCTION

Among 29 plant virus groups, potyviruses are considered the largest and economically most important group (Hamilton et al. 1981; Hollings and Brunt, 1981). The viruses consist of single-stranded, positive-sense RNA encapsidated in flexuous rod-shaped particles which have normal lengths of 680-900 nm (Hollings and Brunt, 1981). One of the features of this virus group is the occurrence of various proteinaceous inclusions in infected host tissue (Edwardson, 1966; 1974; Christie and Edwardson, 1977). Among these inclusions, the cytoplasmic cylindrical inclusions (CI) which contain protein monomers with molecular weight (Mr.) of 67-70k (Hiebert and McDonald, 1973) have long been recognized as a taxonomical characteristic of potyviruses (Edwardson, 1966; 1974; Hamilton et al., 1981). Two other types of inclusions are amorphous inclusions (AI) and nuclear inclusions (NI), which also have been associated with the infections caused by some potyviruses (Christie and Edwardson, 1977). The AI associated with papaya ringspot virus (PRSV) and pepper mottle virus (PeMV) have been purified and characterized (de Mejia et al., 1985a; 1985b). The AI induced by PRSV contain one type of protein monomer with Mr. of 51k and it is serologically related to helper component, a soluble protein which is responsible for aphid transmission of some potyviruses (de Mejia et al., 1985a; 1985b). Similar results were

obtained with PeMV-AI, but the AI proteins of the two viruses are distinct, though related. The NI associated with tobacco etch virus (TEV) infections are the only NI that have been characterized. The TEV-NI consists of two types of protein monomers with Mr. of 54k and 49k, respectively (Knuhtsen et al., 1974; Dougherty and Hiebert, 1980b). The CI, AI and NI proteins have all been shown to be virus specific, host independent and encoded in the respective viral genome (Purcifull et al., 1973; Dougherty and Hiebert, 1980b; de Mejia et al., 1985a; 1985b). Genome maps of potyviruses have been proposed based on the immunoprecipitations of in vitro translation products of potyviral RNA using antisera to those inclusion proteins and capsid protein (Dougherty and Hiebert, 1980a). Antiserum against 54k NI protein of TEV reacts with in vitro translation products, similar in size to TEV 54k NI protein, of all other potyviruses (23 different viruses) which have been translated in the rabbit reticulocyte lysate (RRL) system (E. Hiebert, unpublished data). This indicates that the serological properties of 54k NI protein are very conserved among different potyviruses and implies that an important biological function may be associated with the 54k NI protein. However, the in vivo occurrence of proteins related to TEV 54k NI protein has not been documented for most potyviruses. It would be difficult to resolve the biological significance associated with the 54k NI protein unless more information about NI induced by viruses other than TEV is explored and accumulated.

It has been reported that clover yellow vein virus (CYVV) and some strains of bean yellow mosaic virus (BYMV) induce inclusions in

nuclei of infected tissue (Christie and Edwardson, 1977; Nagel et al., 1983). So far, nothing is known about the characteristics of these NI and their relations to TEV NI.

The purpose of this study was to resolve the properties of the NI associated with BYMV and CYVV infections by purifying them from infected tissue and by characterizing the proteins associated with them. The ultimate objectives are to relate this information to what we already know about TEV NI and consequently to understand what the NI and their proteins contribute to the biology of potyviruses.

CHAPTER 2
PURIFICATION, CHARACTERIZATION AND IMMUNOLOGICAL ANALYSIS OF NUCLEAR INCLUSIONS INDUCED BY BEAN YELLOW MOSAIC VIRUS AND CLOVER YELLOW VEIN VIRUS

Introduction

It is well known that potyviruses are capable of inducing several different kinds of nonstructural proteins in infected cells (Matthews, 1982). Some of these proteins aggregate to form distinct structures known as inclusion bodies, such as amorphous inclusions (AI), cylindrical inclusions (CI) and nuclear inclusions (NI), in different parts of the infected cells (Edwardson, 1974). Another nonstructural protein, helper component, however, was found existing as a soluble form in infected cells (Thornbury and Pirone, 1983). All these proteins except NI proteins have been purified and characterized from at least two different potyviruses. The CI, for example, were found consistently associated with infections of potyviruses (Edwardson, 1966; 1974; Christie and Edwardson, 1977) and all contain a single species of 67-73k protein monomer (Hiebert and McDonald, 1973; Hiebert et al., 1984). On the contrary, although NI are associated with infections of several members of the potyvirus group (Edwardson, 1974; Christie and Edwardson, 1977) only the NI induced by tobacco etch virus (TEV) have been purified and characterized (Knuhtsen et al., 1974; Dougherty and Hiebert, 1980b). The NI induced by TEV contain

two species of protein monomers with Mr. of 54k and 49k, respectively. Antiserum to TEV 54k NI reacts with in vitro translation products of all other potyviruses which have been translated in the rabbit reticulocyte lysate system (E. Hiebert, unpublished data). This indicates that the antigenic properties of the large NI protein are very conserved among different potyviruses. This conserved nature might indicate that this NI protein has some important biological functions.

We have isolated and characterized the NI induced by bean yellow mosaic virus (BYMV) and clover yellow vein virus (CYVV), which have been reported to induce NI in infected tissues (Christie and Edwardson, 1977; Nagel et al., 1983). In this chapter, various characteristics of the NI and NI proteins induced by BYMV and CYVV and the serological detection of NI protein antigen in infected tissues are described. Comparisons between NI proteins induced by BYMV, CYVV and TEV also are presented.

Materials and Methods

Culture of Viruses

An isolate (PV-2) of BYMV (Schroeder and Provvidenti, 1966) and an isolate (CYVV-P) of CYVV (Pratt, 1969), kindly provided by F. W. Zettler, were used throughout the study. Both viruses were cultured in pea (Pisum sativum L. 'Alaska') and maintained at a constant temperature of approximately 26 C in a growth room. To avoid the possibility of contamination with each other, Nicotiana benthamiana was periodically used to ascertain the purity of CYVV-P since

BYMV-PV-2 does not infect Nicotiana benthamiana. Sap inoculation was used to transfer viruses from plant to plant.

Partial Purification of NI

Generally, the format of the procedures for purification of tobacco etch virus (TEV) NI developed by Knuhtsen et al. (1974) was followed. However, due to differences in the shape, size and protein content between the NIs, the procedures for purifying NI from tissues infected with BYMV-PV-2 or CYVV-P were carefully evaluated. The preparations from each step and from different treatments were saved and analyzed by SDS-PAGE to determine the most suitable treatments.

'Alaska' pea leaves systematically infected by BYMV-PV-2 were harvested 3-4 weeks after inoculation. The leaves, chilled 1-2 hr in the cold room before blending, were homogenized in a Waring blender for 2 min with 3 ml of sodium phosphate buffer (100 mM, pH 7.2, containing 0.2% sodium sulfite) per gram of tissue. The homogenate was filtered through four layers of cheesecloth and one layer of Miracloth. The filtrate was treated with 5% Triton X-100 by stirring in the cold room for 1.5-2 hr, followed by centrifuging at 2,000 x g for 10 min in a GSA rotor. The pellet was resuspended with resuspending buffer (RB) (20 mM sodium phosphate buffer, pH 8.2, containing 0.5% sodium sulfite) and centrifuged again at 2,000 x g for 10 min. After resuspending the pellet with RB, Triton X-100 was added to a concentration of 2% and the mixture was stirred in the cold room for 1 hr. The preparation was centrifuged at 2,000 x g for 10 min and the pellet was resuspended with RB containing 20% sucrose. This preparation was homogenized in a Sorvall Omnimixer at position #7 for

1 min and then subjected to a sucrose step gradient (50%:60%:80% sucrose in RB) centrifugation at 7,000 x g for 12 min. The fractions containing 60% and 80% sucrose were collected, diluted with RB and centrifuged at 3,000 x g for 15 min. The pellet was resuspended with a small volume of 20 mM Tris-HCl, pH 8.2 (2 ml/100 g tissue). Hereafter, this preparation will be referred to as BYMV-PV-2 semipurified NI.

To monitor the distribution of NI proteins during purification, preparations saved from each step were dissociated by adding equal volumes of Laemmli dissociation buffer (100 mM Tris-HCl, pH 6.8, 2.5% SDS, 5% 2-mercaptoethanol (ME) and 5% sucrose) and heating in a boiling water bath for 3 min. The samples were loaded in a 7.5-15% gradient gel and electrophoresed at constant 60 V for 16 hr (Hiebert et al., 1984). The acrylamide gel and running buffer were prepared as described by Laemmli (1970). Protein bands were visualized by staining with Coomassie blue R-250 or silver.

Purification procedures for CYVV-P induced NI were similar to those for BYMV-PV-2 NI except sucrose step gradient centrifugation was omitted and the centrifugation force used was greater than that for BYMV-PV-2 NI.

'Alaska' pea tissue systemically infected with CYVV-P was collected 15-20 days after inoculation, chilled 1-2 hr in the cold room and homogenized in a Waring blender with 3 ml of sodium phosphate buffer, as described above, per gram of tissue. The homogenate was filtered through four layers of cheesecloth and one layer of Miracloth. The filtrate was treated with 5% Triton X-100 and stirred

1.5-2 hr in the cold room, followed by centrifuging at 164 x g for 10 min. The supernatant was centrifuged at 16,300 x g for 10 min and the pellet was resuspended with RB. The sample was centrifuged again at 16,300 x g for 10 min and then treated with 2% Triton X-100 for 1 hr, followed by centrifuging at 16,300 x g for 10 min. The pellet was resuspended in a small volume of 20 mM Tris-HCl, pH 8.2 (4 ml/100 g tissue). This preparation will be referred to hereafter as CYVV-P semipurified NI.

Further Purification by Gel Electrophoresis

The proteins of semipurified NI were further purified by preparative gel electrophoresis as described by Hiebert et al. (1984). Semipurified NI from CYVV-P and BYMV-PV-2 were dissociated by adding an equal volume of Laemmli dissociation buffer and heating in a boiling water bath for 3 min before loading on a 7.5-15% gradient preparatory gel. The gel and running buffer were prepared as described by Laemmli (1970). Samples were electrophoresed at a constant 55 V for 16 hr. The inclusion protein bands were visualized by soaking the gel in cold 200 mM KCl and excised with a sharp razor blade. The excised bands were washed three times with deionized water for 5 min each and then frozen at -20 C for at least 2 hr. To ensure the purity of protein, we usually loaded excised protein bands on a second preparatory gel and electrophoresed again under identical conditions. The excised bands were thawed and pressed through a cushion of 1-2 ml of Laemmli dissociation buffer on the top of a stacking gel. The protein bands were carefully loaded to avoid trapping of air bubble between the stacking gel and the protein

bands. After electrophoresis the protein band was visualized and excised as described above. If required, the protein band could be re-electrophoresed again without any appreciable loss of protein during manipulation. The protein was eluted from the gel as described by Hiebert et al. (1984) with modifications. The frozen protein gel was crushed with a mortar and pestle and an appropriate amount of distilled, deionized water was added as elution medium. This material was incubated overnight at room temperature and then centrifuged at 1,000 x g for 5 min. The pellet was reextracted by adding 2 x volume of distilled deionized water and incubated for another 4 hr. After centrifuging at 1,000 x g for 5 min, the supernatants were combined and filtered through a millipore membrane (pore size, 0.45 um), and then water was removed by freeze-drying. The dried material was reconstituted with 2-3 ml of distilled deionized water and dialyzed overnight against distilled deionized water. Protein yields were estimated by spectrophotometry at A 280 and the proteins were stored at -20 C. Purity of protein was determined by analysis in SDS-PAGE and visualization by silver staining.

Purification of Cylindrical Inclusion Protein and Virus Capsid Proteins

Cylindrical inclusions and viruses of CYVV-P and BYMV-PV-2 were purified as described by Nagel et al. (1983) except that Hepes buffer (20 mM, pH 8.2, containing 0.01 M EDTA) was used to resuspend virus preparations. The CI protein and capsid protein of CYVV-P and BYMV-PV-2 were further purified by preparatory gel electrophoresis as described above.

Antisera Preparation

Antisera to NI, CI and capsid proteins were prepared in New Zealand white rabbits as described by Hiebert et al. (1984). Purified antigen was adjusted to 1.0 mg in 1.0 ml with distilled deionized water and emulsified with 1.0 ml of Freund's complete adjuvant. Part of the emulsion (0.15 ml) was injected into the rabbits' footpads, while the rest of the emulsion was administered intramuscularly into the hind legs. This injection procedure was repeated two times at weekly intervals but with Freund's incomplete adjuvant substituted for the complete adjuvant. The rabbits were bled at weekly intervals after the final injection. The reactivities of antisera were evaluated routinely by SDS-immunodiffusion tests.

SDS-Immunodiffusion and Indirect ELISA Tests

The SDS-immunodiffusion tests were conducted as described by Purcifull and Batchelor (1977). Purified NI proteins and other proteins were prepared in 0.1% SDS. Crude, SDS-treated antigens were prepared from infected and noninfected 'Alaska' pea leaves by grinding the tissue with water (1 ml per gram tissue) and then adding another 1 ml of 3% SDS. The antigens were freeze-dried and reconstituted with water before use. Undiluted antisera and preimmune sera were added in the wells and the plates were incubated at 25 C for 48-72 hr. When the reaction was completed, the wells were emptied by aspiration and then filled with a suspension of Norit A charcoal (15 g in 100 ml water).

In attempts to detect NI protein antigen in plant tissue, indirect enzyme-linked immunosorbent assays (ELISA) were conducted,

according to the method of Yeh and Gonsalves (1985) with some modifications. Plant tissue was ground and diluted in coating buffer (50 mM sodium carbonate, pH 9.6, and 0.01% sodium azide) and then added to the wells (100 ul/well) of polystyrene microtitration plates (Immulon II, Dynatech Labs, Inc., Alexandria, VA) for overnight incubation at 30 C. Plates were then washed three times (3 min each) with phosphate buffered saline, pH 7.4, containing 0.05% Tween-20 (PBST) and then loaded with 3% bovine serum albumin (BSA) in PBS (150 ul/well) to block the uncoated sites. After blocking for 1-2 hr, the plates were washed two times with PBST and 100 ul of gamma-globulin (IgG) were added to the wells at 1 ug/ml diluted in enzyme buffer (PBST/2% PVP-40/0.2% ovalbumin) and incubated at 30 C for 3 hr. The IgG to NI proteins were prepared by ammonium sulfate precipitation and DEAE column chromatography (Clark and Adams, 1977). After being washed three times with PBST, 100 ul of goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma, A-8025) diluted 1,000 x in enzyme buffer was added and incubated for 3 hr at 30 C. The plates were washed as before and the bound conjugate was detected by adding 150 ul of the substrate p-nitrophenyl phosphate at 1 mg/ml in 100 mM diethanolamine buffer, pH 9.6, and incubating the plates at room temperature. Reactions were recorded by a plate reader (Model EL 307, Bio-tek Inc.) at 405 nm 60 min after addition of the substrate.

Protein Blotting Procedure

Protein blotting was conducted according to the method of Towbin et al. (1979). Proteins were separated in SDS-PAGE and transferred to

nitrocellulose membranes (NCM, 0.45 um pore size, Bio-Rad, Chemical Division, 2200 Wright Ave., Richmond, CA 94804) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol), at 0 C for 5 hr at constant 100 V using a TE transphore-electrophoretic transfer unit (Hoefer Scientific Instruments, San Francisco, CA 94107). After transfer, the NCM was incubated 2 hr or overnight with a blocking solution containing 3% BSA (Sigma A-7030) in Tris buffered saline (TBS, 20 mM Tris, pH 8.2, 0.9% NaCl) to minimize the nonspecific binding of protein probes to the NCM. The NCM was then incubated for 2-3 hr with various antisera which were diluted in TBS containing 0.1% BSA and 1% normal goat serum. After three 15-minute washes with washing buffer (TBS containing 0.1% BSA), goat anti-rabbit IgG alkaline phosphatase (Sigma A-8025) was used at 1/1,500 dilution in washing buffer and incubated with the NCM for 3 hr. After further washing as described above, followed by a 5-min incubation in 1 M diethanolamine buffer (pH 9.6) the immune complex was visualized by incubating the NCM with the development solution. The development solution consisted of 18 ml diethanolamine buffer (1 M, pH 9.6), 2 ml of 1 mg/ml p-nitroblue tetrazolium (NBT, Gibco 870-1180) in diethanolamine buffer, 0.2 ml of 5 mg/ml p-toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate (BCIP, ICN 100368) in diethanolamine buffer and 40 ul of 2 M magnesium chloride. The reaction was performed at room temperature until appropriate color development occurred. Reactions were stopped by rinsing the NCM in water.

Peptide Mapping by Partial Digestion With V-8 Protease

The chemical natures of NI proteins were compared by partial proteolytic digestion with Staphylococcus aureus V-8 protease as described by Cleveland et al. (1977) with some modifications. The digested peptides were diluted in sample buffer (10 mM sodium phosphate, pH 7.2, 7 M urea, 1% SDS, 1% 2-mercaptoethanol) and boiled for 2 min. The acrylamide gel system was prepared according to Bio-Rad's instructions which consisted of a 15% acrylamide resolving gel and a 3.5% acrylamide stacking gel, both prepared in sodium phosphate buffer (100 mM, pH 7.2) containing 0.1% SDS and 6 M urea. The samples were electrophoresed at constant 55 V for 16 hr using 0.1 M sodium phosphate buffer, pH 7.2 containing 0.1% SDS as running buffer. Digested peptides were visualized by silver staining.

Light Microscopy and Immunofluorescence Tests

Presence of NI in infected cells was observed by light microscopy of the epidermal strips from 'Alaska' pea leaves stained with the Luxol brilliant green BL-calcomine orange ZRS technique (Christie and Edwardson, 1977), hereafter referred to as O-G stain. Purification procedures of NI were monitored by light microscopic examination of preparations stained with O-G stain. Immunofluorescence tests were conducted as described by Hiebert et al. (1984) with modifications. The 60 ul of reaction mixture contained 6 ul of 10% dimethyl sulfoxide (DMSO, Sigma D-5879), 6 ul of antiserum, 30 ul of healthy pea extract (10 x in PBS) and 18 ul of PBS. Rhodamine conjugated protein A was used as a fluorescence probe.

In Vitro Translation and Immunoprecipitation Analysis.

In vitro translation, immunoprecipitation of translation product as well as the isolation of RNA for translation were as described by Dougherty and Hiebert (1980c).

Results

Light Microscopy of Nuclear Inclusions

Nuclear inclusions were observed consistently in leaves infected with BYMV-PV-2 and CYVV-P. No inclusions were seen in healthy control tissue. The NI induced by BYMV-PV-2 were morphologically different from those induced by CYVV-P. The former induced cuboidal nuclear inclusions (Fig. 1A). The sizes of the BYMV-PV-2 NI were highly variable (Fig. 1B). Unlike BYMV-PV-2, CYVV-P induced clusters of particle-like NI which were individually much smaller than BYMV-PV-2 NI (Fig. 1C and 1D). Both types of NI were easily stained with O-G stain but not by azure A stain, indicating they were proteinaceous. The BYMV-PV-2 also induced a structure in the cytoplasm, hereafter referred to as cytoplasmic crystals (CC), which were morphologically indistinguishable from the NI (Fig. 1B). No cytoplasmic crystals were seen in healthy tissue or in the tissue infected by CYVV-P.

Purification of NI and NI Proteins

The semipurified NI from BYMV-PV-2, when observed under light microscopy, were cuboidal, square-like from the top view, highly variable in size and indistinguishable from those seen in situ (Fig. 2A). Attempts to observe substructure of these NI by electron microscopy were unsuccessful. Only very darkly stained NI could be

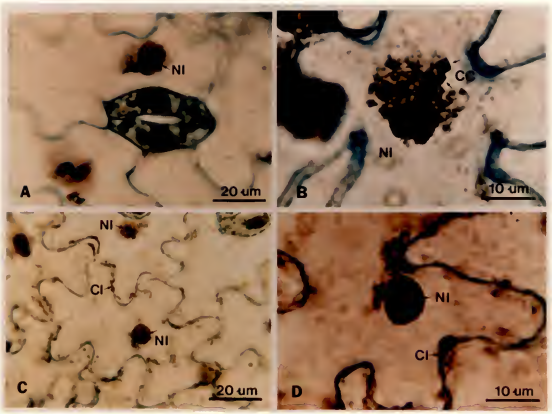


Fig. 1. Light micrographs of infected epidermal tissue of 'Alaska' pea stained with Luxol brilliant green/calcomine orange showing A, BYMV-PV-2 induced nuclear inclusions; B, BYMV-PV-2 induced nuclear inclusions and cytoplasmic crystals; C, CYVV-P induced nuclear inclusion and membrane-associated cylindrical inclusions under low magnification (250 X); D, CYVV-P induced clusters of granular-like nuclear inclusions and cylindrical inclusions under high magnification (500 X). NI, nuclear inclusions; CI, cylindrical inclusions; CC, cytoplasmic crystals.

Fig. 2. Photomicrographs of semipurified nuclear inclusions of BYMV-PV-2 and CYVV-P. A, semipurified BYMV-PV-2 nuclear inclusions stained with Luxol brilliant green/calcomine orange under light microscopy; B, semipurified CYVV-P diamond-shaped nuclear inclusions stained with uranyl acetate under electron microscopy; C, electron micrograph of individual CYVV-P nuclear inclusion.

seen. Unlike BYMV-PV-2, no distinct structure of the semipurified CYVV-P NI could be resolved under light microscopy. Occasionally, very small particulate structures could be barely seen. When exposed to electron microscopy, diamond-shaped structures were easily observed in the semipurified CYVV-P NI preparations (Fig. 2B). No substructure could be resolved on the CYVV-P NI by electron microscopy (e.g., Fig. 2c).

During purification, most of the BYMV-PV-2 NI were sedimented following centrifugation at 2,000 x g (data not shown). By contrast, most of the CYVV-P NI required at least 6,870 x g to be sedimented (data not shown). After sucrose step gradient centrifugation, the majority of the BYMV-PV-2 NI were in the 80% and 60% fractions, while the majority of the CYVV-P NI were in the 50% fraction. These observations were consistent with the findings of the microscopic studies.

When the semipurified NI samples were analyzed in SDS-PAGE, BYMV-PV-2 NI contained two types of protein monomer with molecular weights (Mr.) of 54k and 49k (Fig. 3). These two monomers were roughly estimated to be equimolar based on staining intensity of the protein bands by Coomassie blue R-250 (Fig. 3). A minor 98k protein was consistently associated with the semipurified BYMV-PV-2 NI (Fig. 3). Based on Mr., the BYMV-PV-2 NI protein monomers were identical to those of TEV NI monomers (Fig. 3). Similarly, the CYVV-P NI also contained two different protein monomers, but the Mr. were 60k and 49k (Fig. 3). The 60k and 49k monomers were not in equimolar amounts due to the apparently different staining intensity of these

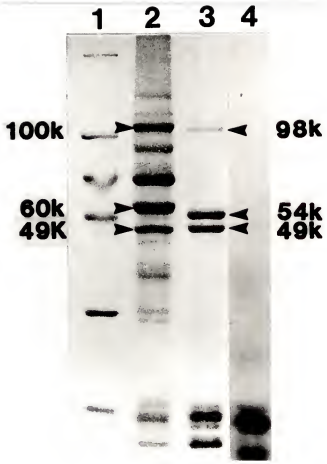


Fig. 3. Analysis of semipurified nuclear inclusions of BYMV-PV-2 and CYVV-P. Lane 1, protein markers; Lane 2, semipurified CYVV-P nuclear inclusions; Lane 3, semipurified BYMV-PV-2 nuclear inclusions; Lane 4, preparation obtained by the same purification procedure from healthy pea tissue. Nuclear inclusion proteins are indicated by arrows. The molecular weights of CYVV-P nuclear inclusion proteins are indicated on the left hand side of the figure while the molecular weights of BYMV-PV-2 nuclear inclusion proteins are indicated on the right hand side of the figure. Marker proteins are tobacco mosaic viral coat protein subunit (17.5k), carbonic anhydrase (29k), glutamin dehydrogenase (53k), bovine serum albumin (67k), phosphorylase b (94k) and myosin (200k).

two proteins in SDS-PAGE. Like BYMV-PV-2 NI, semipurified CYVV-P NI also consistently contained a protein with Mr. of 100k (Fig. 3). The protein monomers of CYVV-P and BYMV-PV-2 NI were further purified successfully by preparatory gel electrophoresis. Due to the closeness between 54k and 49k protein bands in SDS-PAGE and the possible contamination of 53k F1 protein, we routinely purified 54k protein by electrophoresing the excised protein gel four consecutive times in preparatory SDS-PAGE. The BYMV-PV-249k NI protein and CYVV-P NI proteins were always purified twice in preparatory SDS-PAGE, with satisfactory results (Fig. 4).

Immunological Analyses of NI Proteins

Reactive antisera to NI proteins of BYMV-PV-2 and CYVV-P were obtained 2 weeks after the final injections. Reciprocal SDS-immunodiffusion tests showed that the 54k and 49k NI proteins of BYMV-PV-2 were serologically distinct from each other and also distinct from CI protein and capsid protein induced by BYMV-PV-2 (Fig. 5A). Likewise, 60k and 49k NI proteins of CYVV-P were also shown to be serologically distinct from each other, and from CI and capsid proteins of CYVV-P (Fig. 5B). All antisera to NI proteins gave prominant bands when tested with crude plant extracts in SDS-immunodiffusion tests. No reactions were found between these antisera and healthy control plant extracts (Figs. 5 and 6).

Specificity of NI proteins antisera was also evaluated by Western blot analysis. Antisera against CI and capsid protein were also included in the experiments. The results showed that each antiserum specifically recognized its homologous antigen in these tests. No

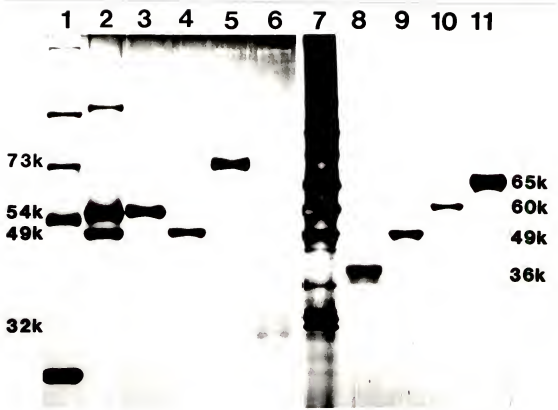


Fig. 4. Analysis of purified nuclear inclusion protein, cylindrical inclusion protein and capsid protein in SDS-polyacrylamide gel electrophoresis. Protein profiles were visualized by silver staining. Lane 1, protein markers; Lane 2, semipurified BYMV-PV-2 nuclear inclusions (NI); Lane 3, purified BYMV-PV-2 54k NI protein; Lane 4, purified BYMV-PV-2 49k NI protein; Lane 5, purified BYMV-PV-2 73k CI protein; Lane 6, purified BYMV-PV-2 32k capsid protein; Lane 7, semipurified CYVV-P NI; Lane 8, purified CYVV-P 36k capsid protein; Lane 9, purified CYVV-P 49k NI protein; Lane 10, purified CYVV-P 60k NI protein; Lane 11, purified CYVV-P 65k CI protein. Molecular weights of purified proteins are indicated on sides of the figure. Protein markers are tobacco mosaic viral coat protein subunit (17.5k), carbonic anhydrase (29k), glutamic dehydrogenase (53k), bovine serum albumin (67k), phosphorylase b (94k) and myosin (200k).

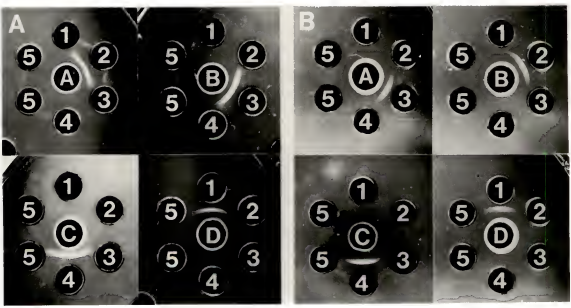


Fig. 5. Two nuclear inclusion proteins of BYMV-PV-2 and CYVV-P were immunologically and serologically distinct from each other and from cylindrical inclusion protein and capsid protein as determined by SDS-immunodiffusion tests. A, reciprocal SDS-immunodiffusion tests of BYMV-PV-2 induced NI proteins, CI protein and CP. Content of center wells were A, BYMV-PV-2 CI antiserum; B, BYMV-PV-2 CP antiserum; C, BYMV-PV-2 49k NI antiserum; D, BYMV-PV-2 54k NI antiserum. Peripheral wells contained PAGE-purified (0.05 mg/ml in 0.1% SDS) BYMV-PV-2 54k NI protein (1), CI protein (2), CP (3), 49k NI protein (4), and SDS-treated healthy 'Alaska' pea tissue extract (5). B, reciprocal SDS-immunodiffusion tests of CYVV-P induced NI proteins, CI protein and CP. Content of center wells were A, CYVV-P CP antiserum,; B, CYVV-P CI protein antiserum; C, CYVV-P 49k NI protein antiserum; D, CYVV-P 60k NI protein antiserum. Peripheral wells contained PAGE-purified (0.05 mg/ml in 0.1% SDS) CYVV-P 60k NI protein (1), CI protein (2), CP (3), 49k NI protein (4) and SDS-treated healthy 'Alaska' pea tissue extract (5).

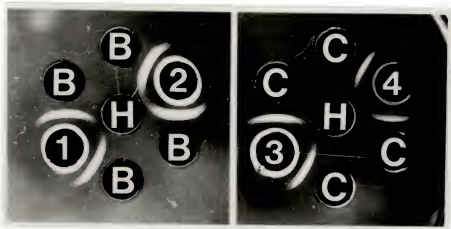


Fig. 6. Detection of nuclear inclusion proteins in infected pea tissue by SDS-immunodiffusion tests. Wells contained 1, BYMV-PV-2 54k NI protein antiserum; 2, BYMV-PV-2 49k NI protein antiserum; 3, CYVV-P 60k NI protein antiserum; 4, CYVV-P 49k NI protein antiserum; B, SDS-treated BYMV-PV-2 infected 'Alaska' pea crude extract; C, SDS-treated CYVV-P infect 'Alaska' pea crude extract; H, SDS-treated healthy 'Alaska' pea crude extract.

cross reactivities were found between any antiserum and heterologous antigens. However, antisera to BYMV-PV-2 NI proteins (e.g., 54k and 49k) reacted both with the 98k protein of BYMV-PV-2 (Fig. 7) and antisera to CYVV-P NI proteins (e.g., 60k and 49k) reacted both with the 100k protein of CYVV-P (Fig. 8). No reactions were observed when normal serum was used (Figs. 7 and 8).

Peptide Mapping of NI Proteins

Distinct peptide patterns were found between 54k and 49k NI proteins of BYMV-PV-2 when these proteins were partially digested with Staphylococcus aureus V-8 protease (Fig. 9). Likewise, peptide patterns of CYVV-P 60k and 49k NI proteins were distinct from each other (Fig. 10). These results showed that NI protein monomers of BYMV-PV-2 and CYVV-P were chemically different.

Immunofluorescence of NI and NI Proteins

Antisera to NI proteins of CYVV-P and BYMV-PV-2 were used in immunofluorescence tests to react with NI in situ. All four antisera induced specific fluorescence with their respective NI in the nuclei (Fig. 11). The morphology of NI detected by immunofluorescence tests was indistinguishable from that found by light microscopy (Fig. 11). Antisera to BYMV-PV-2 54k and 49k NI protein also reacted and showed very strong immunofluorescence with cytoplasmic crystals induced by BYMV-PV-2 (Fig. 11A). No cytoplasmic crystals were observed in CYVV-P infected tissue by light microscopy, but clusters of particles hereafter referred to as granular structures (GS), usually located in the cytoplasm next to the nucleus, were readily detected in immunofluorescence tests using antisera to CYVV-P NI proteins

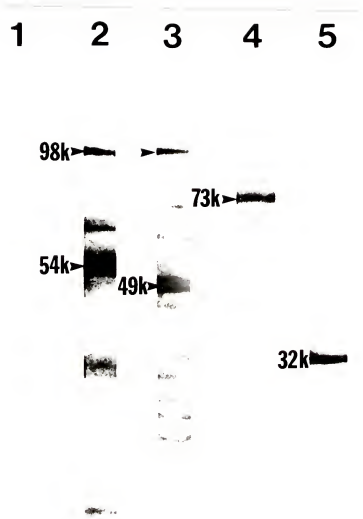


Fig. 7. Western blot analysis of proteins contained in semipurified BYMV-PV-2 nuclear inclusion preparations. After protein transfer, nitrocellulose membrane strips were incubated with Lane 1, normal serum; Lane 2, BYMV-PV-2 54k NI protein antiserum; Lane 3, BYMV-PV-2 49k NI protein antiserum; Lane 4, BYMV-PV-2 CI protein antiserum; Lane 5, BYMV-PV-2 CP antiserum. Resulting antigen-antibody complexes were further detected by incubating with goat anti-rabbit IgG alkaline phosphatase conjugate and visualized by substrate reactions as described in the text. Molecular weights are indicated alongside of each major protein.

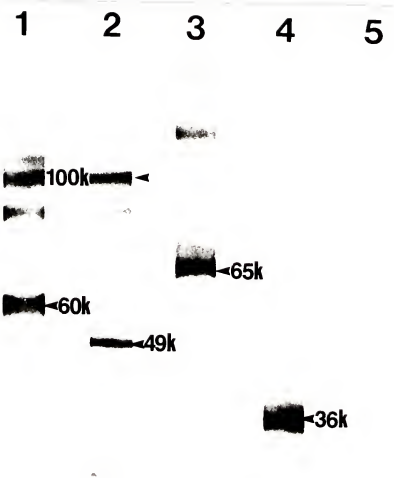


Fig. 8. Western blot analysis of proteins contained in semipurified CYVV-P nuclear inclusion preparations. After protein transfer, nitrocellulose membrane strips were incubated with Lane 1, CYVV-P 60k NI protein antiserum; Lane 2, CYVV-P 49k NI protein antiserum; Lane 3, CYVV-P CI protein antiserum; Lane 4, CYVV-P CP antiserum; Lane 5, normal serum. Resulting antigen-antibody complexes were further detected by incubating with goat anti-rabbit IgG alkaline phosphatase conjugate and visualized by substrate reactions as described in the text. Molecular weights of major proteins are indicated on the sides of each protein.

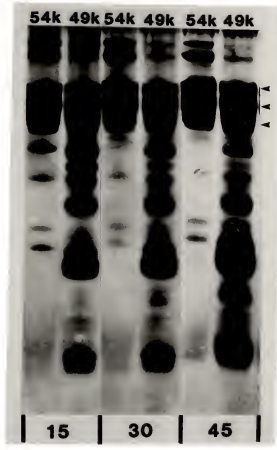


Fig. 9. Staphylococcus aureus V-8 protease digest pattern comparison of PAGE-purified BYMV-PV-2 NI protein monomers. The protease digested peptides were separated by SDS-PAGE and visualized by silver staining. Arrows indicate components of V-8 protease consistently observed in each lane. Identities of proteins are indicated on the top of each lane. Numbers on the bottom indicate digestion time in minutes.

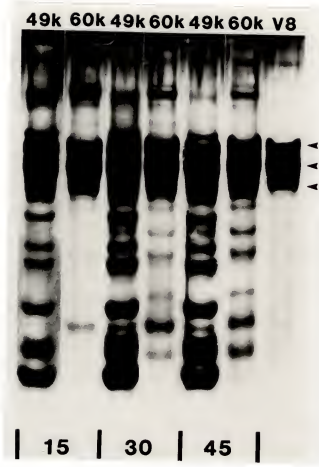


Fig. 10. Staphylococcus aureus V-8 protease digest pattern comparison of PAGE-purified CYVV-P NI protein monomers. The protease digested peptides were separated by SDS-PAGE and visualized by silver staining. Arrows indicate components of V-8 protease consistently observed in each lane. Identities of proteins are indicated on the top of each lane. Numbers on the bottom indicate digestion time in minutes.

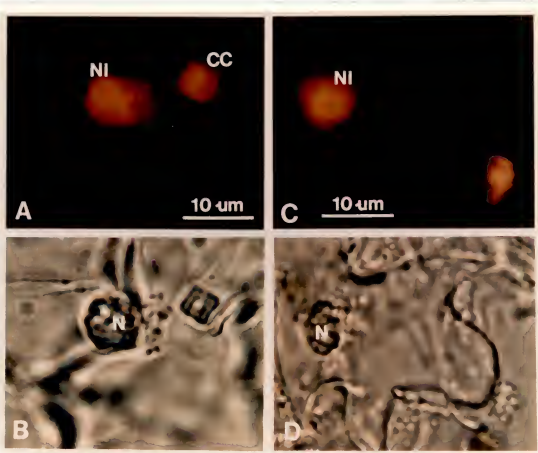


Fig. 11. Detection of nuclear inclusion in situ by immunofluorescence microscopy. A, BYMV-PV-2 infected pea epidermal strip photographed with epifluorescence optics showing the specific fluorescence from NI and cytoplasmic crystal treated with BYMV-PV-2 54k NI protein antiserum; B, the same field of view photographed with visible light; C, CYVV-P infected pea epidermal strip photographed with epifluorescence optics, showing the specific fluorescence from NI treated with CYVV-P 49k NI protein antiserum; D, the same field of view photographed with visible light. NI, nuclear inclusion; CC, cytoplasmic crystal; N, nucleus.

(Fig. 12). These granular structures did not immunofluoresce when CYVV-P CI or capsid protein antisera were used.

Origin of NI and NI Proteins

The observations described above brought up the question concerning the origin of these NI-like cytoplasmic crystals. In attempts to resolve this question, we conducted the following experiments. Epidermal strips of BYMV-PV-2 infected leaves of pea were taken daily, starting 24 hrs after inoculation and processed for immunofluorescence tests using antisera to BYMV-PV-2 49k NI, 54k NI, CI and CP as probes. Normal serum was used as a control to determine nonspecific reactions. We found the plasmalemma of some locally infected cells showed specific immunofluorescence with all four antisera 48 hr after inoculation (Fig. 13). No NI was detected at this time. Nuclear inclusions were only detected 72 hr after inoculation, and at this stage the size of NI was rather small and no cytoplasmic crystals were detected (Fig. 14A and 14B). With time, the number of NI in nuclei and the size of each NI gradually increased (Fig. 14C and 14D). About 10 days after inoculation most of the nuclei were packed with large amounts of NIs (Fig. 14E and 14F). It was at this stage that the cytoplasmic crystals were detected (Fig. 14E and 14F). We believe that these cytoplasmic crystals originated from the NI in the nuclei. They ruptured the nuclear membrane and were released into the cytoplasm. This hypothesis was further tested by observing the tissues stained with O-G stain (Fig. 15). Cytoplasmic crystals were consistently associated with those

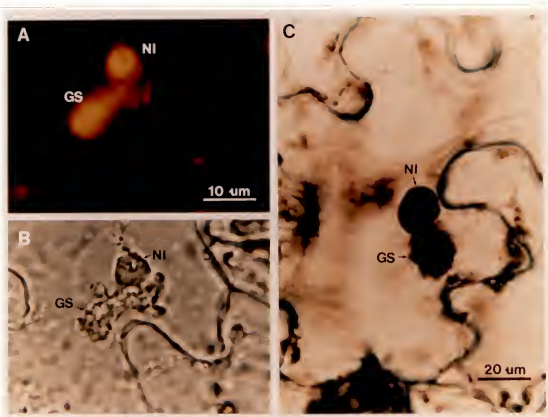
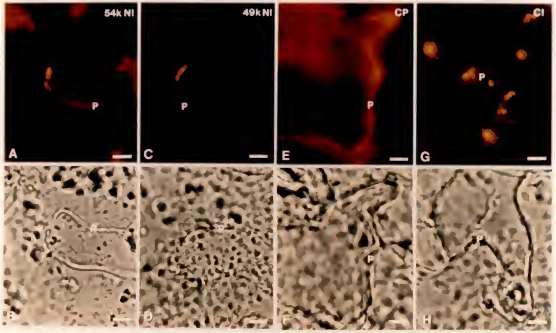


Fig. 12. Detection of CYVV-P induced granular structure in situ by immunofluorescence microscopy and light microscopy. A, CYVV-P infected pea epidermal strip photographed with epifluorescence optics showing the specific fluorescence from NI and granular structure treated with CYVV-P 60k NI protein antiserum; B, the same field of view as in A, but photographed with visible light; C, Light micrographs showing CYVV-P induced NI and granular structure darkly stained by Luxol brilliant green/calcomine orange. Note the similarity of the outline of the GS as detected by immunofluorescence and light microscopy. NI, nuclear inclusion; GS, granular structure.

Fig. 13. Detection of BYMV-PV-2 induced proteins associated with plasmalemma at the very early stage of infection by immunofluorescence microscopy. A, BYMV-PV-2 infected pea epidermal strip 48 hr after inoculation photographed with epifluorescence optics showing specific fluorescence from plasmalemma treated with BYMV-PV-2 54k NI protein antiserum; B, the same field of view photographed with visible light; C, BYMV-PV-2 infected epidermal strip 48 hr after inoculation photographed with epifluorescence optics showing specific fluorescence from plasmalemma treated with BYMV-PV-2 49k NI protein antiserum; D, the same field of view photographed with visible light; E, BYMV-PV-2 infected peak epidermal strip 48 hr after inoculation photographed with epifluorescence optics showing specific fluorescence from plasmalemma treated with BYMV-PV-2 CP antiserum; F, the same field of view photographed with visible light; G, BYMV-PV-2 infected epidermal strip 48 hr after inoculation photographed with epifluorescence optics showing specific fluorescence from plasmalemma treated with BYMV-PV-2 CI protein antiserum; H, the same field of view photographed with visible light. Bar = 5 um.


54k NI
49k NI
CP
CI
P
P
P
P
A
C
E
G

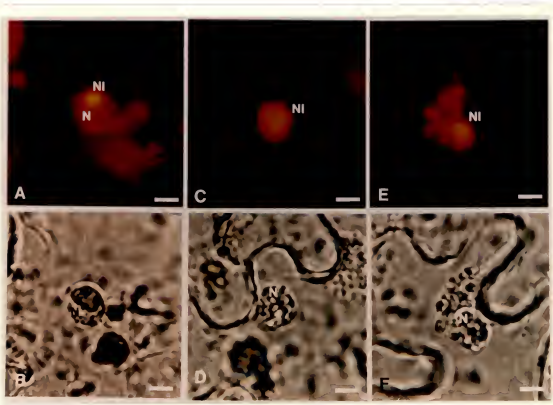


Fig. 14. Time course localization of BYMV-PV-2 nuclear inclusion in situ by immunofluorescence microscopy showing the increase in the number and size of nuclear inclusion and possible release of nuclear inclusion from the nucleus to the cytoplasm. Epidermal strips obtained from lower side of inoculated 'Alaska' pea leaves were treated with either BYMV-PV-2 54k NI protein antiserum or 49k NI protein antiserum and photographed with epifluorescence optics. A, BYMV-PV-2 NI detected 5 days after inoculation; B, the same field of view photographed with visible light; C, BYMV-PV-2 NI detected 10 days after inoculation; D, the same field of view photographed with visible light; E, BYMV-PV-2 NI detected 15 days after inoculation; F, the same field of view photographed with visible light. Bar = 5 um.

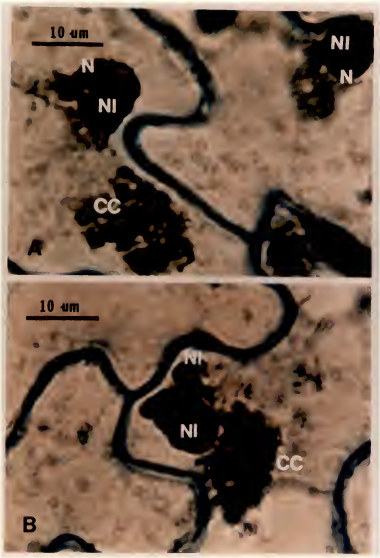


Fig. 15. Light micrographs of BYMV-PV-2 infected epidermal tissue of 'Alaska' pea stained with Luxol brilliant green/calcomine orange showing A, cytoplasmic crystals associated with ruptured nucleus (note the intact nucleus on the upper right corner without cytoplasmic crystals associating with it); B, a nuclear inclusion apparently released from a nucleus packed with nuclear inclusions, a hole on the nuclear membrane can be seen. N, nucleus; CC, cytoplasmic crystals; NI, nuclear inclusions.

cells which had nuclear membranes that were ruptured due to accumulation of NIs. No cytoplasmic crystals were seen in cells with intact nuclei (Fig. 15).

Serological Comparison of NI Proteins Between TEV, CYVV-P and BYMV-PV-2

Reciprocal SDS-immunodiffusion tests were conducted to compare serological properties of NI induced by TEV, CYVV-P and BYMV-PV-2. Using antisera to large NI proteins (e.g., 54k proteins for TEV and BYMV-PV-2, 60k protein for CYVV-P) to react with crude extracts of these three viruses, all three viruses are related to, but distinct from, each other based on the formation of spurs between homologous and heterologous antigens (Fig. 16). On the other hand, if antisera to small NI proteins (e.g., 49k NI proteins) were used, only BYMV-PV-2 and CYVV-P were related to, but still distinct from, each other (Fig. 16). Antiserum to TEV 49k NI protein only reacted with its homologous antigen. Likewise, antisera to 49k NI proteins of BYMV-PV-2 and CYVV-P reacted homologously and heterologously with each other, but did not react to TEV NI at all. This experiment indicated that serological properties of large NI proteins were very conserved among different potyviruses, while those of small NI proteins were more viral-specific. Antigenic conservativeness of large NI proteins was further confirmed by using the antisera to these three different large NI proteins to immunoprecipitate in vitro translation products of pepper mottle virus (PeMV), a virus which was heterologous to any of the antisera used. The results are shown in Fig. 17. All three antisera immunoprecipitated an identical set of products. This result

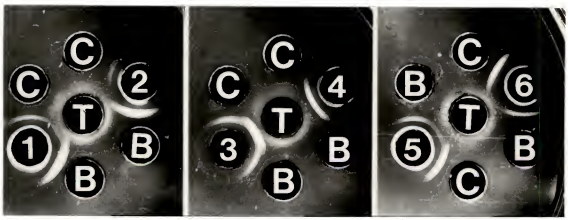


Fig. 16. Serological relationship of large and small nuclear inclusion proteins between TEV, CYVV-P and BYMV-PV-2. Wells contained 1, BYMV-PV-2 54k NI protein antiserum; 2, BYMV-PV-2 49k NI protein antiserum; 3, TEV 54k NI protein antiserum; 4, TEV 49k NI protein antiserum; 5, CYVV-P 60k NI protein antiserum; 6, CYVV-P 49k NI protein antiserum; C, SDS-treated CYVV-P infected 'Alaska' pea extract; B, SDS-treated BYMV-PV-2 infected 'Alaska' pea extract; T, SDS-treated TEV infected Datura stramonium tissue.

Fig. 17. Protein profiles of in vitro translation products of BYMV-PV-2 RNA and PeMV RNA immunoprecipitated with antisera to the large NI proteins of BYMV-PV-2, CYVV-P and TEV, showing the serological conservativeness of these three large NI proteins. Translations of BYMV-PV-2 RNA (Lane 1-3) and PeMV RNA (Lane 4-6) were done in rabbit reticulocyte lysate system as described in the text. The figure illustrates [S^{35}]methionine-labeled proteins immunoprecipitated by each antiserum were separated in 7.5-15% SDS-PAGE and detected by fluorography. Lanes 1 and 4, products immunoprecipitated by antiserum to BYMV-PV-2 54k NI protein; Lanes 2 and 5, products immunoprecipitated by antiserum to TEV 54k NI protein; Lanes 3 and 6, products immunoprecipitated by antiserum to CYVV-P 60k NI protein.

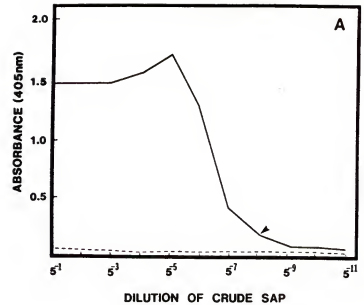
was again in agreement with previous findings (E. Hiebert, unpublished data).

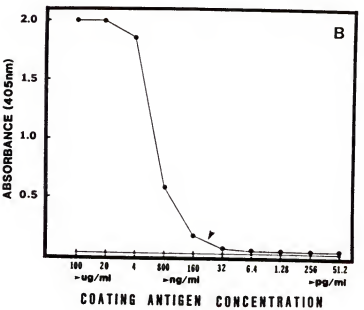
Detection of 54k NI Related Protein in Tissue Infected by Potyviruses

The 54k NI protein was chosen because, as mentioned, this protein represents a conservative, potyviral specified protein which might be valuable as a target for indexing potyviral infections. Indirect ELISA was used as the major technique to detect 54k NI related protein in tissue infected by different potyviruses. Our objective was to determine if all potyviruses induced sufficient amounts of this type of protein in infected tissue, so that this approach could be applied as a potyvirus indexing method on a regular basis.

Immunoglobulin was isolated from antiserum to 54k NI protein of BYMV-PV-2 and used in the indirect ELISA tests. Crude sap of pea tissue infected with BYMV-PV-2 could be diluted up to 10×5^8 times and still gave positive reactions (Fig. 18). When purified BYMV-PV-2 54k NI protein was used, it was readily detected at a concentration of 32-160 ng/ml (Fig. 18). In general, this technique is very sensitive in detecting 54k NI protein in crude sap without any background problems (Fig. 18). Several potyviruses, including CYVV, watermelon mosaic virus-2 (WMV-2), cowpea aphid-borne mosaic virus (CAMV), peanut mottle virus (PMoV), TEV, bean yellow mosaic virus (BYMV-204-1), BYMV-Scott and BYMV isolate from orchid, were detected inducing this antigen in infected tissue using this system. However, no reactions were obtained with tissue infected with papaya ringspot virus type P

Fig. 18. Detection of 54k NI protein antigen in BYMV-PV-2 infected pea tissue by indirect ELISA. A, crude sap from BYMV-PV-2 infected 'Alaska' pea tissue extracted and diluted in coating buffer by 5-fold series were used as coating antigen; B, PAGE-purified BYMV-PV-2 54k NI protein at the initial concentration of 100 ug/ml diluted with healthy crude sap extracted in (5 x) volume of coating buffer were used as coating antigen. After coating, the uncoated sites on plates were saturated with 3% BSA. Immunoglobulin to 54k NI protein was used at 1 ug/ml and goat anti-rabbit IgG alkaline phosphatase conjugate at 1/1,000 dilution. Readings were recorded 1 hr after adding substrate at 1 mg/ml. ⊢——⊣, readings of purified 54k NI protein; ⊢——⊣, readings of healthy control pea extract; ———, readings of BYMV-PV-2 infected pea extract; - - - - - , reading of healthy pea extract. Arrows indicate dilution end points where positive reactions were obtained.


A
ABSORBANCE (405nm)
2.0
1.5
1.0
0.5
5^{-1}
5^{-3}
5^{-5}
5^{-7}
5^{-9}
5^{-11}
DILUTION OF CRUDE SAP


B
ABSORBANCE (405nm)
2.0
1.5
1.0
0.5
100 20 4 800 160 32 6.4 1.28 256 51.2
←ug/ml
←ng/ml
←pg/ml
COATING ANTIGEN CONCENTRATION

(PRSV-P), PRSV-W, blackeye cowpea mosaic virus (BlCMV), pepper mottle virus (PeMV) and peanut stripe virus (PStV).

Discussion

Nuclear inclusions induced by BYMV-PV-2 and CYVV-P were successfully purified. Due to differences in size, shape and protein content of the NI induced by different viruses, the success of purification depended on careful evaluation, step by step, of the treatments used. Generally, we found NI were very resistant to Triton X-100 treatment, a property which facilitated their purification. The NI could be retreated with Triton X-100 several times and separated from the green pigment of the plant extracts by low speed centrifugation. A similar approach has been used in amorphous inclusions purifications (de Mejia et al., 1985a).

As with TEV NI (Knuhtsen et al., 1974), the NI of BYMV-PV-2 and CYVV-P consist of two species of protein monomers. This seems to be a common phenomenon for NI induced by potyviruses. The two protein monomers in each NI may be equimolar as with TEV (Knuhtsen et al., 1974) and BYMV-PV-2, or existing in different ratios as with CYVV-P NI. The NI induced by BYMV-PV-2 consistently contained a protein with Mr. of 98k. Likewise, a 100k protein was always associated with the NI induced by CYVV-P. This was also consistent with the well known phenomenon associated with TEV NI (Knuhtsen et al., 1974; Purcifull and Hiebert, 1982; Hiebert et al., 1984). The 95k protein associated with the TEV NI was thought to be a dimer of the two protein monomers (Hiebert et al., 1984). However, in the case of BYMV-PV-2 and CYVV-P,

these high Mr. proteins reacted with both the antisera against the two protein monomers, indicating these proteins might be polyproteins. Similar results have been obtained in a study of in vivo proteins associated with TEV infection (Hari and Abdel-Hak, 1985). Additional evidence for the polyprotein nature of the 98-100k proteins will be presented in the next chapter.

Using antisera to TEV NI proteins, Dougherty and Hiebert (1980b) were able to show antisera to 54k and 49k NI proteins each immunoprecipitated a different set of in vitro translation products of TEV RNA. In this chapter, we demonstrated that the two NI protein monomers were antigenically distinct from each other and from CI and CP by SDS-immunodiffusion tests and Western blotting. Evidence was also presented showing that the two NI protein monomers were chemically different by separating the proteolytically digested peptides. In the next chapter, we will illustrate that the two protein monomers of BYMV-PV-2 were coded in BYMV-PV-2 genome.

Antisera to NI proteins could be used in immunofluorescence tests to detect NI in situ despite the fact that SDS-treated NI proteins were used as the immunogens. This not only provided evidence that the isolated NI proteins were the major constituent of the NI seen in situ, but also indicated the SDS-treated proteins still preserved, at least in part, the antigenicities of the natural proteins. Similar results have been consistently obtained with antisera prepared to potyviral CI, AI and TEV NI (de Mejia et al., 1984a; Hiebert et al., 1984).

The NI induced by TEV could easily be resolved under electron microscopy showing a distinctive cross-hatched substructure (Knuhtsen et al., 1974; Purcifull and Hiebert, 1982). Attempts to observe substructures of BYMV-PV-2 and CYVV-P NI were unsuccessful. This was probably because these NI were cubical-shaped, instead of a thin plate-like structure as TEV NI. The thickness of these NI prevented the electron beam from penetrating, thus no substructures could be resolved.

As mentioned, the antigenic properties of the TEV 54k NI protein were possibly very conserved among different potyviruses due to the broad spectrum reactivities of its antiserum to the in vitro translation products of more than 20 potyviruses (E. Hiebert, unpublished data). We demonstrated here that 54k NI protein of TEV, 54k NI protein of BYMV-PV-2 and the 60k NI protein of CYVV-P, all representing the large monomers of NI, were serologically related. The fact that all three antisera to the large monomer of three different NI could immunoprecipitate an identical set of peptides from in vitro translation of a heterologous viral RNA, further confirmed the serological conservativeness of the large monomers of potyviral NI. Unfortunately, it was found that not every potyvirus induced enough of this type of antigen in tissue extracts to permit its detection by indirect ELISA. Therefore, it does not seem feasible to use this type of protein as a target for indexing potyviral infection. If potyviruses, as proposed by most workers, possessed a polyprotein type of translational strategy (Vance and Beachy, 1984; Allison et al., 1985; Dougherty et al., 1985; Yeh and Gonsalves,

1985), then they should theoretically induce equal amounts of every protein that they encode. The reason why some potyviruses did not induce sufficient quantities of 54k type antigen in tissue is still unknown. It is highly possible that some potyviruses induce this type of protein which exists as soluble form in the infected cells, but it does not normally aggregate into inclusion bodies. This soluble protein may easily be degraded by cellular proteases of plant origin and consequently could not be detected. Further experiments, like the time course appearance study, could be applied to solve this question.

We have done the time course appearance study of BYMV-PV-2 NI and shown that the plasmalemma was possibly the site of potyviral protein synthesis at the very early stage of infection. We also demonstrated that cytoplasmic crystals usually found in BYMV-PV-2 infected tissue were actually NI-like in nature. These types of crystals were probably the same as the structures, known as dense bodies, frequently found in tissue infected by various BYMV strains by electron microscopy (Christie and Edwardson, 1977; Carr and Kim, 1983). To confirm this, immunological techniques using colloidal gold particle labelled probes to study the ultrathin sections of BYMV infected tissue under EM could be applied in further experiments. Although no cytoplasmic crystals were observed in CYVV-P infected tissue, granular structures usually located adjacent to the nucleus in the CYVV-P infected cells were probably NI-like as well, since they reacted with CYVV-P 60k and 49k NI protein antisera but did not react with CI and CP antisera in the immunofluorescence tests. It is also possible that these granular structures, similar to cytoplasmic crystals of BYMV,

represent NI that are released from the nucleus due to large accumulation of NI and consequent rupturing of the nuclear membrane.

CHAPTER 3
GENOME MAPPING OF BEAN YELLOW MOSAIC VIRUS AND EVIDENCE FOR PROTEOLYTICAL PROCESSING OF POLYPROTEINS AS THE TRANSLATION STRATEGY OF THE VIRUS

Introduction

It has been well documented that cylindrical inclusions (CI), nuclear inclusions (NI), amorphous inclusions (AI) and helper component (HC) are nonstructural proteins of potyviral origin (Dougherty and Hiebert, 1980a; 1980b; Hellmann et al., 1983; Hiebert et al., 1984; de Mejia et al., 1985a; 1985b). Sequences coding for these nonstructural proteins along with the sequences encoding capsid protein (CP) have been mapped in the potyviral genome by immunological analysis of the in vitro translation products of potyviral RNA (Dougherty and Hiebert, 1980a; de Mejia et al., 1985b). Based on analysis of in vitro translation of tobacco etch virus (TEV) and pepper mottle virus (PeMV) RNA in a rabbit reticulocyte lysate (RRL) system, Dougherty and Hiebert (1980a) first proposed a genome map with CP located near the 3'-terminus and a 78-87k unknown gene near the 5'-terminus. This unknown gene was further identified as containing two genes, with the gene encoding amorphous inclusion or helper component located toward the 3'-terminus and a 30-60k gene toward the 5'-terminus, based on analysis of in vitro translation products of papaya ringspot virus (PRSV) and PeMV RNA in both the wheat germ (WG)

system and RRL system (de Mejia et al., 1985b). Location of the capsid protein gene of TEV and PeMV at the 3'-terminus of their genome has been shown by direct sequencing of a clone of cDNA and a fragment of RNA, corresponding to the CP genes of TEV and PeMV (Allison et al., 1985; Dougherty et al., 1985). That the TEV 54k NI protein gene was adjacent to the CP gene of TEV was also confirmed by direct sequencing of the same fragment of cDNA (Allison et al., 1985). It was proposed that sequences coding for 49k NI, CI and a unknown gene product were located in the center part of the potyviral genome (Dougherty and Hiebert, 1980a). However, the actual order of these three genes in relation to 54k NI and AI was still uncertain due to a lack of evidence to link any of these three genes with AI or 54k NI (E. Hiebert, personal communication). Yeh and Gonsalves (1985) found that deletion of dithiothreitol (DTT) from the RRL system could inhibit the proteolytical processing activity of a lysate factor, which required a thio-group for reactivity, and resulted in the occurrence of several high molecular weight proteins in the in vitro translation products of PRSV RNA. With the addition of DTT in the translation medium, high molecular weight proteins could not be detected. This system, by adjusting the incorporation of DTT, might allow the detection of more high molecular proteins or polyproteins which have not been found before and could help to resolve the order of genes located in the central part of the potyviral genome. In this chapter, we will present evidence resolving the order of genes in bean yellow mosaic virus RNA using the system described above.

Evidence has accumulated in recent years that the proteolytical processing of polyproteins translated from full-length genomic RNA is the major translation strategy of potyviruses (Hellmann et al., 1980; Dougherty, 1983; Vance and Beachy, 1984; Yeh and Gonsalves, 1985). In a sequencing study of the TEV capsid protein gene, direct evidence of polyprotein synthesis and processing was given showing that no stop codon existed in between sequences coding for CP and 54 NI of TEV. Consequently, CP and 54k NI were first synthesized into a polyprotein and later proteolytically processed at a glutamine-glycine site into individual proteins (Allison et al., 1985). However, the presence of a definitive polyprotein in vivo has not been demonstrated. In Chapter 2, we illustrated that a 98k protein associated with semipurified NI of BYMV-PV-2 and a 100k protein from semipurified CYVV-P NI were possibly polyproteins because they were immunoreactive with antisera to either small or large nuclear inclusion protein monomers. In this chapter, we will present detailed evidence showing the polyprotein nature of these two proteins. The presence of these polyproteins in relation to the translation strategy and the order of NI genes in the genome of potyviruses will also be discussed.

Materials and Methods

Culture of Viruses

The BYMV-PV-2 and CYVV-P sources and propagation methods were described in Chapter 2.

Virus Purification

The BYMV-PV-2 and CYVV-P were purified as described by Nagel et al. (1983) except that Hepes buffer (20 mM, pH 8.2, containing 10 mM EDTA) was used to resuspend virus preparations throughout the purification procedure.

Purification of 98k and 100k Proteins

The 98k protein induced by BYMV-PV-2 and 100k protein induced by CYVV-P were purified by the methods for purifying NI of BYMV-PV-2 and CYVV-P as described in Chapter 2.

Antisera Preparation, SDS-Immunodiffusion, and Western Blotting Analysis

Antisera to 98k protein of BYMV-PV-2 and 100k protein of CYVV-P were prepared as described in Chapter 2. The SDS-immunodiffusion tests were performed according to the methods developed by Purcifull and Batchelor (1977). Purified proteins were adjusted to appropriate concentrations with 0.1% SDS for reaction. Western blotting procedures were described in Chapter 2.

RNA Isolation

Freshly purified virions in Hepes buffer (20 mM, pH 8.2, containing 10 mM EDTA) were dissociated by adding an equal volume of dissociation buffer (200 mM Tris, 2 mM EDTA, 2% SDS, pH 9.0) and incubated at 60 C for 2 min. The mixture was centrifuged in a linear-log sucrose density gradient (Brakke and Van Pelt, 1970a) and the fractions containing 39 S RNA were collected. The RNA was precipitated by the addition of sodium acetate (pH 5.0, final concentration 100 mM) and 2 vol of 100% ethanol. After incubation at

-20 C overnight, the RNA was collected by low speed centrifugation (12,100 x g, 20 min) and resuspended in a small volume of sterilized water. The concentration of RNA was determined spectrophotometrically, using an extinction coefficient of 25 as 1 ug/ml. The RNA was used immediately or stored at -80 C for later uses.

Lysate Preparation and In Vitro Translation Conditions

The in vitro translation procedures and conditions for rabbit reticulocyte lysate were the same as described by Dougherty and Hiebert (1980c). Rabbit reticulocyte lysate was obtained from Green Hectares (Oregon, WI 53575). The wheat germ (WG) system was prepared as described by Marcu and Dudock (1974). The reactions for the WG system were carried out as described by Salerno-Rife et al. (1980). Eight micrograms of RNA in 100 ul rabbit reticulocyte lysate mixture and 20 ug of RNA in 100 ul of wheat germ mixture, both containing 30-50 uCi of [S^{35}]methionine, were incubated (30 C, 90 min) for reactions. Incorporation of [S^{35}]methionine into polypeptides was assayed by trichloroacetic acid (TCA) precipitation on Whatman 3MM filter paper discs (Mans and Novelli, 1961).

Immunoprecipitation Analysis of Translation Products

Immunoprecipitation analysis was performed as described by Dougherty and Hiebert (1980c). Products immunoprecipitated were separated by 7.5-15% linear-gradient SDS-PAGE and detected on dried gels by fluorography (Bonner and Lasky, 1974). The antisera used were CP, CI, 54k NI and 49k NI proteins of BYMV-PV-2 and CP, CI, 60k NI and 49k NI proteins of CYVV-P. Antisera to tobacco vein mottling virus

(TVMV) helper component (HC), obtained from Dr. D. W. Thornbury, was also used.

Results

Isolation of 98k Protein From BYMV-PV-2 Infected Tissue and 100k Protein From CYVV-P Infected Tissue

As previously described, the 98k protein and 100k protein were found consistently associated with the purified NI preparations of BYMV-PV-2 and CYVV-P, respectively. These two proteins were further purified by preparatory gel electrophoresis. To ensure the purities of these proteins, we routinely electrophoresed the proteins twice consecutively on preparatory gels. The purities of 98k protein and 100k protein were confirmed by analyzing with a 7.5-15% linear gradient SDS-PAGE and visualization of protein bands by silver staining (Fig. 19).

Reconstitution Experiments

It was originally believed that the 98k protein of BYMV-PV-2 and the 100k protein of CYVV, like the 95k protein of TEV NI, were dimers that resulted from incomplete dissociation during SDS-PAGE of the two NI protein monomers. However, it was found that when the 98k and 100k proteins were isolated, re-treated with Laemmli dissociation solution and re-analyzed in SDS-PAGE they still maintained the same Mr. This indicated that those proteins were not the dimers of any of the NI proteins. To test this further, we also put purified 54k NI and 49k NI proteins of BYMV-PV-2 together without adding any dissociation or reducing agents and analyzed the mixture in SDS-PAGE after 4 hr of

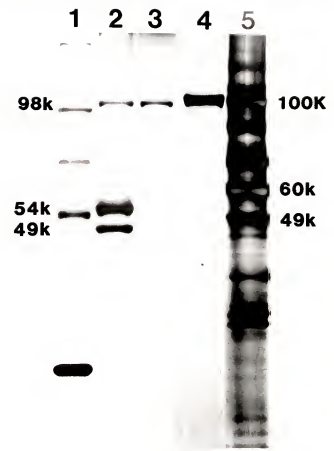


Fig. 19. Analysis of purified 98k and 100k protein in SDS-PAGE. Proteins were visualized by silver staining. Lane 1, protein markers; Lane 2, semipurified BYMV-PV-2 nuclear inclusions; Lane 3, purified BYMV-PV-2 98k protein; Lane 4, purified CYVV-P 100k protein; Lane 5, semipurified CYVV-P nuclear inclusions. Molecular weights of proteins are indicated on sides of the figures. Protein markers are tobacco mosaic viral coat protein subunit (17.5k), carbonic anhydrase (29k), glutamic dehydrogenase (53k), bovine serum albumin (67k), phosphorylase b (94k) and myosin (200k).

incubation (Fig. 20). The results showed that no high Mr. protein occurred under these conditions, indicating that chances for formation of dimers of either 54k or 49k protein due to incomplete dissociation were minor. Another experiment was set up also to confirm the above conclusion. The BYMV-PV-2 NI were purified through the same procedures with 1.0%, 0.5% or no reducing agent (sodium sulfite) in the working buffer. The NI preparations thus obtained were analyzed in SDS-PAGE and no differences were found in protein profiles between different treatments (Fig. 20). This indicated that the occurrences of BYMV-PV-2 98k protein were not likely to be due to oxidation and later dimer formation of 54k or 49k proteins during purification.

Immunological Analysis of 98k and 100k Proteins

Antisera were prepared against purified 98k and 100k proteins. Reactive antiserum to 100k protein was obtained 1 week after the final injection, whereas antiserum against 98k protein was not reactive until 3 weeks after the final injection. The reactivity of the two antisera were tested first in SDS-immunodiffusion tests. The data are shown in Fig. 21. Antiserum to the 100k protein of CYVV-P reacted with antigens of 60k NI protein, 49k NI protein and homologous 100k protein, but not with CI protein, CP and healthy extract from pea tissue. Antiserum to the 98k protein of BYMV-PV-2, however, reacted only with antigens of 49k NI protein and its homologous 98k protein of BYMV-PV-2. It did not react with 54k NI protein, CI protein, CP and healthy pea tissue extract. Antisera to 60k NI and to 49k NI of CYVV-P both reacted with 100k protein antigen. Likewise, antisera to

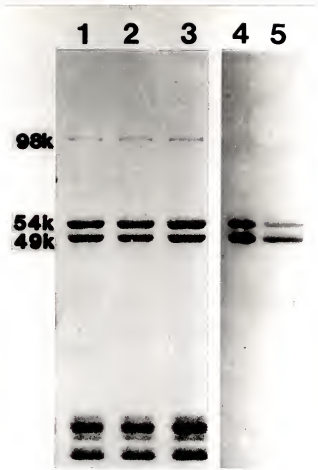
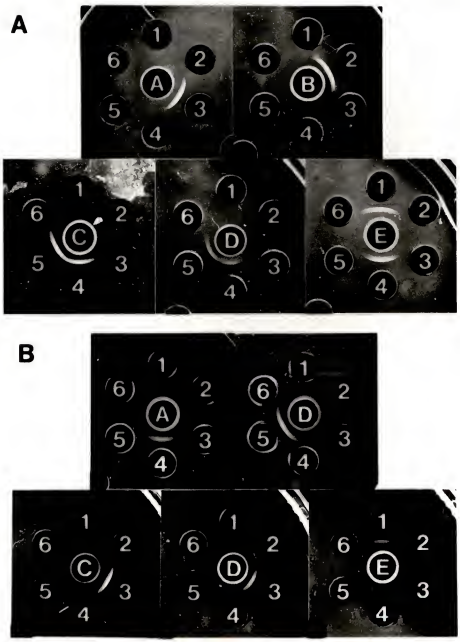


Fig. 20. A reconstitution experiment using BYMV-PV-2 54k NI and 49k NI protein and analysis of semipurified BYMV-PV-2 nuclear inclusion preparations obtained by isolating with different concentration of sodium sulfite. Proteins were separated in 7.5-15% linear gradient SDS-PAGE and visualized with Coomassie blue staining. Lane 1, semipurified nuclear inclusion preparation obtained using sodium phosphate buffer (0.01 M, pH 7.2) without sodium sulfite; Lane 2, semipurified nuclear inclusion preparation obtained using same buffer containing 0.5% sodium sulfite; Lane 3, semipurified nuclear inclusion preparation obtained using same buffer containing 1.0% sodium sulfite; Lane 4, 20 ug of 54k NI protein in 20 ul water was mixed with 20 ug of 49k NI protein in 20 ul water and analyzed in SDS-PAGE after 4 hr of incubation; Lane 5, 10 ug of 54k NI protein in 10 ul water was mixed with 10 ug of 49k NI protein in 10 ul water and analyzed in SDS-PAGE after 4 hr of incubation. Molecular weights of nuclear inclusion proteins are indicated on the sides of the figure.

Fig. 21. Immunological analysis of BYMV-PV-2 98k protein and CYVV-P 100k protein in SDS-immunodiffusion tests. A, reciprocal SDS-immunodiffusion tests of BYMV-PV-2 induced 98k protein, two NI proteins, CI protein and CP. Contents of center wells were A, BYMV-PV-2 CI protein antiserum; B, BYMV-PV-2 CP antiserum; C, BYMV-PV-2 49k NI protein antiserum; D, BYMV-PV-2 98k protein antiserum; E, BYMV-PV-2 54k NI protein antiserum. Peripheral wells contained PAGE-purified (0.05 mg/ml in 0.1% SDS) BYMV-PV-2 54k NI protein (1), CP (2), CI protein (3), 98k protein (4), 49k NI protein (5) and SDS-treated healthy pea tissue extract (6). B, reciprocal SDS-immunodiffusion tests of CYVV-P induced 100k protein, NI proteins, CI protein and CP. Content of center wells were A, CYVV-P CI protein antiserum; B, CYVV-P CP antiserum; C, CYVV-P 49k NI protein antiserum; D, CYVV-P 100k protein antiserum; E, CYVV-P 60k NI protein antiserum. Peripheral wells contained PAGE-purified (0.05 mg/ml in 0.1% SDS) CYVV-P 60k NI protein (1), 100k protein (2), 49k NI protein (3), CI protein (4), CP (5) and SDS-treated healthy pen tissue extract.


A
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54k NI and to 49k NI protein of BYMV-PV-2 both reacted to 98k protein antigen. Based on these results, it is postulated that the 100k protein and 98k protein are polyproteins that contain sequences of both large NI and small NI proteins.

To test this further, we compared the protein profiles obtained by immunoprecipitating the in vitro translation products of BYMV-PV-2 RNA with all the NI antisera used above and the antisera to 100k and 98k proteins. Results thus obtained were identical to those obtained by SDS-immunodiffusion tests (Fig. 22). Antiserum to 98k protein of BYMV-PV-2 gave an identical protein profile to that immunoprecipitated by BYMV-PV-2 49k NI antiserum. It did not immunoprecipitate any proteins specific to 54k NI antiserum. In contrast, 100k protein antiserum immunoprecipitated not only proteins specific to 60k NI antiserum but also proteins specific to 49k NI antiserum. This experiment confirmed the results obtained in SDS-immunodiffusion tests indicating the 100k protein of CYVV-P consisted of immunogenically active epitopes from both 60k and 49k NI proteins of CYVV-P, while the 98k protein, although containing sequences of 54k and 49k proteins, possessed only immunogenically active epitopes from 49k NI protein of BYMV-PV-2. These epitopes from 54k protein contained in 98k protein were not immunogenically active.

Similar results were obtained when testing these antisera in Western blotting analysis of semipurified NI preparations. Antisera to 100k protein of CYVV-P reacted with 60k NI, 49k NI and its homologous 100k protein (Fig. 23). Antisera to both 60k NI and 49k NI

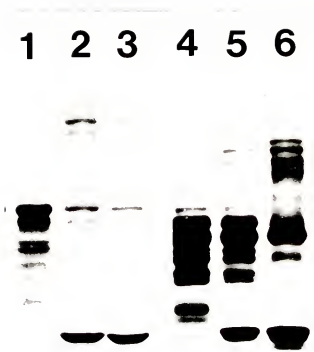


Fig. 22. Immunological analysis of BYMV-PV-2 98k protein and CYVV-P 100k protein by immunoprecipitating in vitro translation products of BYMV-PV-2 RNA with antisera to these two proteins. Translation was done in the rabbit reticulocyte lysate system. Immunoprecipitated [S^{35}]methionine-labeled proteins were separated in SDS-PAGE and visualized by fluorography. Lane 1, products immunoprecipitated with antiserum to BYMV-PV-2 54k NI protein; Lane 2, products immunoprecipitated with antiserum to BYMV-PV-2 98k protein; Lane 3, products immunoprecipitated with antiserum to BYMV-PV-2 49k NI protein; Lane 4, products immunoprecipitated with antiserum to CYVV-P 60k NI protein; Lane 5, products immunoprecipitated with antiserum to CYVV-P 100k protein; Lane 6, products immunoprecipitated with CYVV-P 49k NI protein.

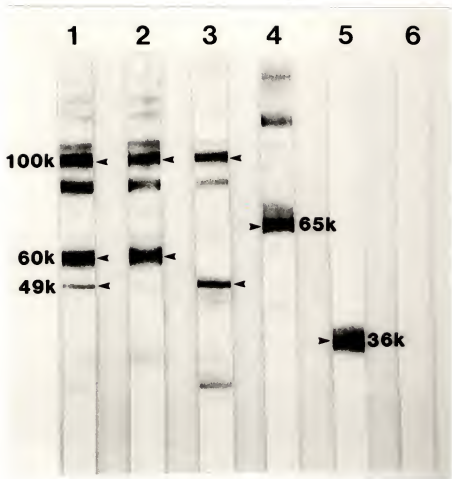


Fig. 23. Immunological analysis of CYVV-P 100k protein by Western blotting. After protein transfer, nitrocellulose membrane strips were incubated with Lane 1, CYVV-P 100k protein antiserum; Lane 2, CYVV-P 60k NI protein antiserum; Lane 3, CYVV-P 49k NI protein antiserum; Lane 4, CYVV-P CI protein antiserum; Lane 5, CYVV-P antiserum; Lane 6, normal serum. Resulting antigen-antibody complexes were detected by incubating with goat anti-rabbit IgG alkaline phosphatase conjugate and visualized by substrate reactions as described in the text. Molecular weights of major proteins are indicated alongside each protein.

of CYVV-P reacted with 100k protein and their homologous antigens (Fig. 23). Similarly, 54k NI and 49k NI antisera of BYMV-PV-2 both reacted with 98k protein and their homologous antigens (Fig. 24). The 98k protein antiserum reacted strongly with 49k NI and homologously to 98k protein but only slightly with 54k NI (Fig. 24). The reactivity of the 98k protein antiserum in Western blotting indicated that this antiserum did contain a very small fraction of antibodies specific to 54k NI protein, although the antiserum did not react with 54k NI protein in SDS-immunodiffusion and immunoprecipitation tests.

Peptide Mapping of 100k and 98k Proteins

In order to determine if the 100k and 98k proteins actually contained sequences of both the large NI and small NI proteins, we performed partial digestion of NI proteins of CYVV-P and BYMV-PV-2 and compared their profiles with the protein profiles of 100k and 98k proteins after protease digestion under the same conditions. In the case of CYVV-P, the 100k protein contained peptides identical to those from 60k and 49k NI proteins of CYVV-P (Fig. 25). The 98k protein of BYMV-PV-2 contained all the peptides from the 54k NI protein but contained seven of the ten peptides generated from the 49k NI protein (Fig. 26). The reason why three of the ten peptides from the 49k NI protein were not detected was probably due to the theoretically lower concentrations of the same peptides that contained in the polyproteins than those contained in normal proteins when both proteins (e.g., polyprotein and normal protein) were prepared at the same concentration.

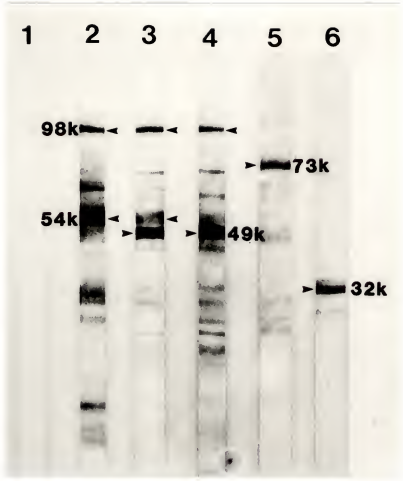


Fig. 24. Immunological analysis of BYMV-PV-2 98k protein by Western blotting. After protein transfer, nitrocellulose membrane strips were incubated with Lane 1, normal serum; Lane 2, BYMV-PV-2 54k NI protein antiserum; Lane 3, BYMV-PV-2 98k protein antiserum; Lane 4, BYMV-PV-2 49k NI protein antiserum; Lane 5, BYMV-PV-2 CI protein antiserum; Lane 6, BYMV-PV-2 CP antiserum. Resulting antigen-antibody complexes were further detected by incubating with goat anti-rabbit IgG alkaline phosphatase conjugate and visualized by substrate reactions as described in the text. Molecular weights of major proteins are indicated on the sides of each protein.

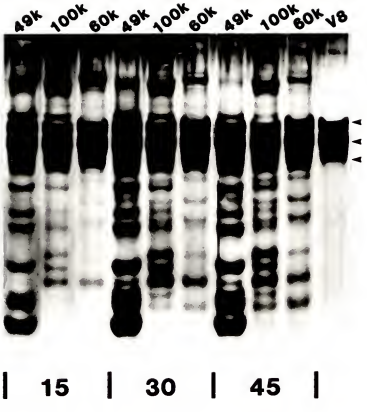


Fig. 25. Staphylococcus aureus V-8 protease digest pattern comparison of CYVV-P 100k protein to CYVV-P 60k and 49k NI proteins. The protease digested peptides were separated by SDS-PAGE and visualized by silver staining. Arrows indicate components of V-8 protease consistently observed in each lane. Identities of proteins are indicated on the top of each lane. Numbers on the bottom indicate digestion time in minutes.

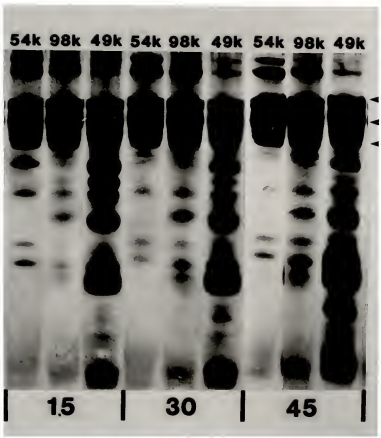


Fig. 26. Staphylococcus aureus V-8 protease digest pattern comparison of BYMV-PV-2 98k protein to BYMV-PV-2 54k and 49k NI proteins. The protease digested peptides were separated by SDS-PAGE and visualized by silver staining. Arrows indicate components of V-8 protease consistently observed in each lane. Identities of proteins are indicated on the top of each lane. Numbers on the bottom indicate digestion time in minutes.

Immunological Analysis of In Vitro Translation Products Obtained in the Rabbit Reticulocyte Lysate (RRL) System

The genomic organization of BYMV-PV-2 was studied by in vitro translation and immunoprecipitation. The translational products obtained in the absence of DTT were compared to the products obtained in the presence of DTT. The results are shown in Fig. 27. A product with Mr. greater than 200k, immunoprecipitated by all antisera except HC, was detected in the lysate translations done without DTT. Occasionally, a product with Mr. considerably greater than the 200k product was found in the same lysate (Fig. 28). Due to a lack of available protein markers greater than 200k, the Mr. of these high molecular weight proteins could not be calculated accurately. Products of 135k, 125k and 117k, immunoprecipitated by antisera to 49k NI, 54k NI and CP, were also found in the absence of DTT. These three products, as well as the products greater than 200k, disappeared and several lower Mr. products appeared 1-2 hr after the addition of DTT to the translation medium. Products with Mr. of 120k, 140k, 150k and 170k, immunoprecipitated only by antisera to 49k NI and CI, were found in lysate translations with and without DTT. A product with Mr. of 85k and a series of smaller Mr. products were immunoprecipitated by antisera to 54k NI and CP, indicating the sequences of these two viral proteins were linked. A 115k product and a 73k product comigrating with the authentic CI were found to be immunoprecipitated only by antiserum to CI. This 115k product was considered as a polyprotein containing sequences encoding an unknown 42k protein and the

Fig. 27. Analysis of in vitro translation products of BYMV-PV-2 RNA by immunoprecipitation. The figure illustrates [S^{35}]methionine-labeled products separated in 7.5-15% SDS-PAGE and detected by fluorography. Translations were done with (Lanes 3, 10-14) and without (Lanes 2, 4-9) incorporation of dithiothreitol (DTT). Lane 1, C^{14} standard proteins. Molecular weight markers in kilodaltons are from the bottom to the top: lysozyme (14.3k), carbonic anhydrase (29k), ovalbumin (46k), bovine serum albumin (67k), phosphorylase b (93k) and myosin (200k). Lane 2, total products of translation done without DTT; Lane 3, total products of translation done with DTT; the following lanes were products immunoprecipitated with antiserum to normal serum (lane 4), to BYMV-PV-2 CI protein (lanes 5 and 10), to BYMV-PV-2 CP (lanes 6 and 11), to BYMV-PV-2 54k NI protein (lanes 7 and 12), to BYMV-PV-2 49k NI protein (lanes 8 and 13), to TVMV HC protein (lanes 9 and 14). Some important products, which were used to construct the genomic map, are identified by arrows and their molecular weights are indicated on the sides of the figure.

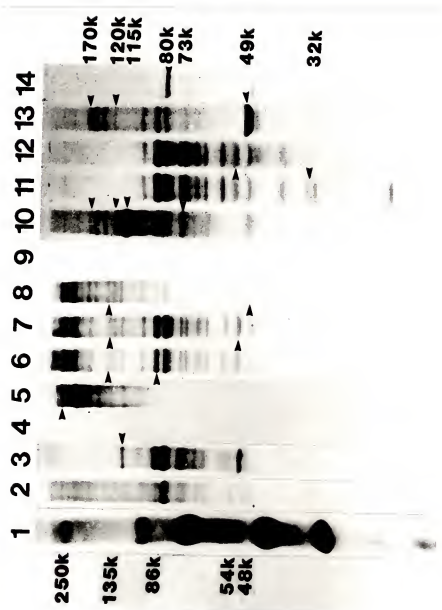
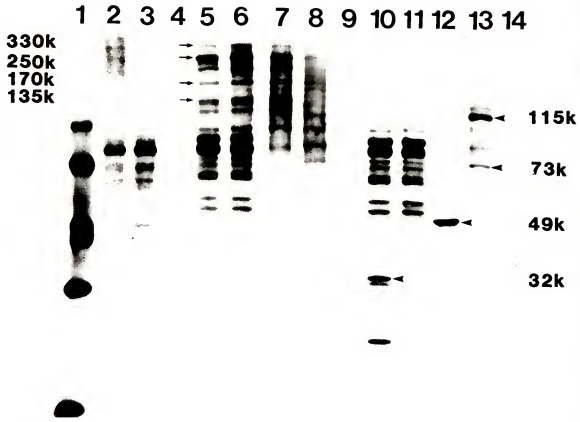

1 2 3 4 5 6 7 8 9 10 11 12 13 14
250k
135k
86k
54k
48k
170k
120k
115k
80k
73k
49k
32k

Fig. 28. Analysis of in vitro translation products of BYMV-PV-2 RNA by immunoprecipitation. The figure illustrates [S^{35}]methionine-labeled products separated in 7.5-15% SDS-PAGE and detected by fluorography. Translations were carried out for 90 min in the absence of DTT. The reaction mixture was further incubated for another 2 hr with (Lanes 3, 10-14) or without (Lanes 2, 4-9) DTT added. Lane 1, C^{14} standard proteins. Molecular weight markers in kilodaltons are from the bottom to the top: lysozyme (14.3k), carbonic anhydrase (29k), ovalbumin (46k), bovine serum albumin (67k), phosphorylase b (93k) and myosin (200k). Lane 2, total products of translation done and further incubated without DTT; Lane 3, total products of translation done without DTT but further incubated with DTT for 2 hr; the following lanes were products immunoprecipitated with antiserum to normal serum (lane 4), to BYMV-PV-2 CP (lanes 5 and 10), to BYMV-PV-2 54k NI protein (lanes 6 and 11), to BYMV-PV-2 49k NI protein (lanes 7 and 12), to BYMV-PV-2 CI protein (lanes 8 and 13), to TVMV HC protein (lanes 9 and 14). Products that disappeared upon addition of DTT were indicated by arrows with tail, while products that appeared after incubation with DTT are indicated by arrows without tail. Molecular weights of important products are indicated on the sides of the figure.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
330k
250k
170k
135k
115k
73k
49k
32k

73k CI protein. Antisera to TVMV HC immunoprecipitated an 80k product, corresponding to the major product of this RRL translation system, and another product with Mr. of 48k. Cross reactivities of products to two or more antisera were confirmed by sequentially immunoprecipitating with two or more different antisera (data not shown). Based on analysis of these translation products, the genome map of BYMV-PV-2 from the 5'-terminus to the 3'-terminus is proposed as follows: 32k unknown protein, 48k HC, 42k unknown protein, 73k CI, 49k NI, 54k NI and 32k CP (Figs. 29 and 30). The precursor and product relationship and the proteolytic processing pathway is also proposed (Fig. 30). Due to a lack of evidence supporting linkages of HC and 42k unknown protein, the organization of this particular part of BYMV-PV-2 genome is not yet conclusive.

Immunological Analysis of In Vitro Translation Products in the Wheat Germ System

As in the previous experiment in the RRL system, translations of BYMV-PV-2 RNA in the WG system were done with and without addition of DTT. Translation products were analyzed by immunoprecipitating with the same set of antisera as used in the RRL system. No differences in product profiles were found between the WG lysates with or without DTT added; however, it was found that the protein profiles between the WG (Fig. 31) and the RRL system were nearly identical. Both lysate translations resulted in major products of 80k, 48k and 32k, but the [S^{35}] signals obtained for these three products were much stronger in the WG system than in the RRL system (Fig. 31). The major 80k and 48k products were immunoprecipitated by antiserum to HC, as in the RRL

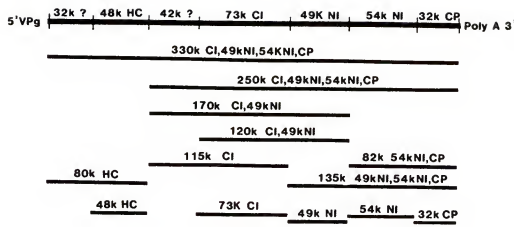


Fig. 29. Interrelationships of products detected in BYMV-PV-2 stimulated rabbit reticulocyte lysate with the proposed genome map of BYMV-PV-2. This map was constructed based on the original map proposed by Dougherty and Hiebert (1980a) with the internal portion re-orientated. Molecular weights of the components and the antisera used for immunoprecipitation are indicated above each product. CI, cylindrical inclusion protein; NI, nuclear inclusion protein; CP, capsid protein; HC, helper component protein; ?, unknown proteins; Poly A, poly A tail at the 3'-terminus of potyviral genome; VPg, genome-linked protein at the 5'-terminus of potyviral genome.

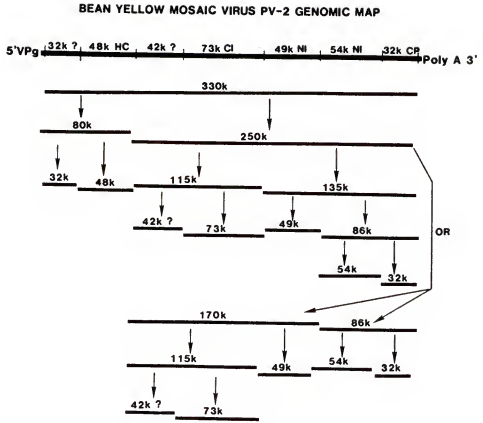
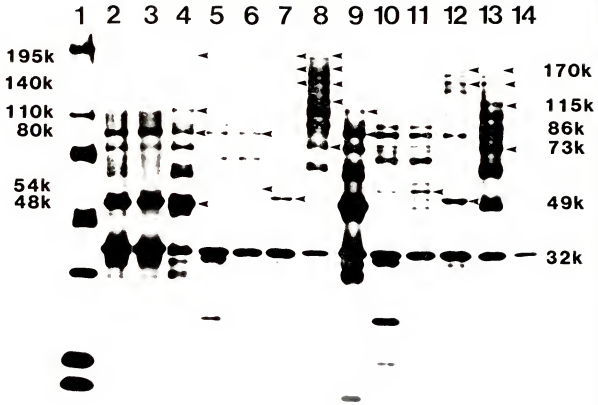


Fig. 30. Proposed genomic map of bean yellow mosaic virus (BYMV-PV-2) and proteolytic processing pathway of in vitro translation products of BYMV-PV-2 RNA in rabbit reticulocyte lysate. The precursor and product relationship and the processing pathway was indicated by placing the smaller products immediately below the precursors. CI, cylindrical inclusion protein; NI, nuclear inclusion protein; CP, capsid protein; HC, helper component protein; ?, unknown proteins; Poly A, poly A tail at the 3'-terminus of potyviral genome; VPg, genome-linked protein at the 5'-terminus of potyviral genome.

Fig. 31. Analysis of in vitro translation products in BYMV-PV-2 RNA stimulated wheat germ lysate by immunoprecipitation. The figure illustrates [S^{35}]methionine-labeled products separated in 7.5-15% SDS-PAGE and detected by fluorography. Translations were carried out with (lanes 3, 9-14) and without (lanes 2, 4-8) DTT incorporated. Lane 1, C^{14} standard proteins. Molecular weight markers in kilodaltons are from the bottom to the top: lysozyme (14.3k), lactoglobulin (18.4k), chymotrypsinogen (2.57k), ovalbumin (43k), bovine serum albumin (68k), phosphorylase b (7.4 k) and myosin (200k). Lane 2, total products of translation done without DTT; Lane 3, total products of translation done with DTT; the following lanes were products immunoprecipitated with antiserum to TVMV HC protein (lanes 4 and 9), to BYMV-PV-2 CP (lanes 5 and 10), to BYMV-PV-2 54k NI protein (lanes 6 and 11), to BYMV-PV-2 49k NI protein (lanes 7 and 12), to BYMV-PV-2 CI protein (lanes 8 and 13) and to normal serum (lane 14). Some important products, which were used to construct the genomic map, are identified by arrows and their molecular weights are indicated on the sides of the figure.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
195k
140k
110k
80k
54k
48k
170k
115k
86k
73k
49k
32k

system. Two other products with Mr. of 110k and 72k, which were not detected in the RRL system, were also immunoprecipitated by antiserum to HC. The 32k product was immunoprecipitated by all the antisera used, but these precipitations were considered as nonspecific reactions because (1) no differentiation between the intensities of 32k product immunoprecipitated by different antisera were observed and (2) even preimmune serum also precipitated this product, a situation that has never been observed before. Based on these observations, the 32k product was considered as an unknown product unrelated to any known potyviral proteins isolated so far. Two products with Mr. of 190k and 185k, immunoprecipitated by antisera to HC, CI and 49k NI, were found in WG lysates with and without DTT. Products with Mr. of 170k, 150k, 140k and 120k, identical to those detected in the RRL system, were found immunoprecipitated by antisera to 49k and CI in the WG system. On the basis of the immunoreactivities and Mr. estimation of the products detected in the WG system, we tentatively proposed another possible genome organization (Fig. 32). The general order of the known genes in this map is the same as that proposed (Fig. 30); however, two possible orders for the unknown gene in the internal portion of BYMV genome are presented.

Discussion

Association of a higher Mr. protein with the two NI monomers is a common phenomenon for the three NI that have been isolated so far (Knuhtsen et al., 1974; Dougherty and Hiebert, 1980; Chang et al., 1985b). Recently the same phenomenon was also observed in the NI

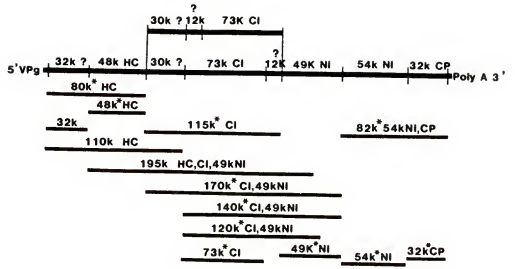


Fig. 32. Interrelationship between products detected in BYMV-PV-2 RNA stimulated wheat germ lysates and the genome map consequently constructed. Two possible orientations of genes in the internal portion of the BYMV-PV-2 genome are presented. Molecular weights of the products and by which antiserum or antisera the products were immunoprecipitated are indicated on the top of each product. Products which were detected in both rabbit reticulocyte and wheat germ lysate are marked with an asterisk. CI, cylindrical inclusion protein; NI, nuclear inclusion protein; CP, capsid protein; HC, helper component protein; ?, unknown proteins; Poly A, poly A tail at the 3'-terminus of the genome; VPg, genome-linked protein at the 5'-terminus of the genome.

isolated from BYMV-204-1 (data not shown). These higher Mr. proteins were originally considered as dimers that presumably resulted from incomplete dissociation of either the large NI or small NI proteins during SDS-PAGE (Hiebert et al., 1984). With the evidence given in the reconstitution experiments showing that these high Mr. proteins appeared independently of the presence of reducing agents, and that they were resistant to further dissociation treatments, these proteins should be considered as separate entities. We further demonstrated that these proteins consisted of sequences from both the large NI and small NI proteins, based on immunological analyses and by mapping the partially digested peptides. These studies provide direct evidences for the polyprotein nature of these proteins.

Antisera prepared against 98k protein of BYMV-PV-2 only reacted with small NI protein (49k NI) but not with large NI protein (54k NI) of BYMV-PV-2 in SDS-immunodiffusion and immunoprecipitation tests. However, it did react slightly with 54k NI protein in Western blot analysis, indicating this antiserum did contain a small quantity of antibodies specific for 54k NI protein. This small quantity of antibodies was only detected by Western blotting because the signal was enhanced by enzyme-substrate reactions during immunoblotting procedures. Possible reasons why the 98k protein antiserum was not very immunoreactive to 54k NI protein are (1) epitopes of the 49k NI were exposed on the surface of the 98k protein and therefore were more accessible during the immune response of the animal, while most epitopes of 54k NI were hidden inside of the polypeptide chain and (2)

the individual rabbit used was genetically unable to respond to most epitopes of the 54k NI protein.

The significance of the existence of these unprocessed polyproteins in the infected tissue is still unknown to us. However, detection of these proteins in vivo indicated that proteolytical processing of polyproteins is part of the translation strategy of potyviruses. Knowing that these polyproteins contained sequences of both NI proteins also implicated the linkage of the two NI protein genes in the potyviral genome.

With deletion of DTT from the RRL system, our results were consistent with those of Yeh and Gonsalves (1985), in that a product with Mr. greater than 200k and a very high Mr. product, possibly corresponding to the 330k product of PRSV, were found in translation lysate without DTT. We further demonstrated that these high Mr. products were readily cleaved once DTT was added. Similar results were also obtained in a study of peanut mottle virus (PMoV) in vitro translations (Xiong et al., 1985). The fact that high Mr. translational products were converted to lower Mr. products following addition of DTT to the translation medium, implicated the proteolytical processing of polyproteins as the translation strategy of BYMV-PV-2 RNA. This suggestion is also consistent with the detection of large amount of nonstructural proteins in the BYMV-PV-2 infected tissue (Chapter 2).

The wheat germ system has been used for in vitro translation of many potyviruses (de Mejia et al., 1935b; Quiot-Douine et al., 1986; E. Hiebert and W. G. Dougherty, unpublished data). Unlike the RRL

system, the template activity of the potyviral genome in the wheat germ system appears to be confined to the two genes proximal to the 5'-terminus (E. Hiebert and W. G. Dougherty, in press). However, the translation of PMoV RNA was the exception that translation product profiles in the WG system were nearly identical to those in the RRL system (Xiong et al., 1985). Our results, similar to those with PMoV, showed that both lysate systems gave identical product profiles. However, in our experiments, the signals obtained for the products translated from the 5'-terminus were much stronger than those from the 3'-terminus. This was shown by the detection of large amounts of 80k and 48k products, immunoprecipitated by antiserum to HC, and the 32k unknown product presumably encoded by the first gene in the 5'-terminus. In addition, antiserum to HC immunoprecipitated a series of products from the WG translation lysate, while these products were not detected in the RRL systems. These are all indications that in the WG system the template proximal to the 5'-terminus was more active than that proximal to the 3'-terminus. The differences in template activity between the two translation systems is not yet understood. Another difference between these two translation systems that we found was that DTT had no effect on the cleavage of high Mr. products into low Mr. products in the WG system because no differences in product profiles were found between the WG translation lysates with or without DTT. It is possible that the ATP, along with DTT required for the activity of primary cleavage factor as suggested by Pelham (1979), was normally incorporated in the WG system used, and it alone could

promote the activity of polyprotein processing without the need of DTT.

The high Mr. products, commonly found in lysates of in vitro translation, were now considered as polyproteins (Dougherty, 1983; Yeh and Gonsalves, 1985) which was very useful in constructing the genome organization of potyviruses based on their immunoreactivity to different antisera against viral proteins (Dougherty and Hiebert, 1980; Hellmann et al., 1983; de Mejia et al., 1985; Xiong et al., 1985). With deletion of DTT from RRL translation medium, we were able to detect some of the high Mr. proteins which have never been documented before, thus allowing construction of a genome map of BYMV-PV-2 RNA. The order for the three genes coded for by the central part of the genome was re-oriented from the original map proposed by Dougherty and Hiebert (1980). This was based on the detection of a 135k product, immunoprecipitated by antisera to 49k NI, 54k NI and CP, and a series of four products with Mr. of 120k, 140k, 150k and 170k, immunoprecipitated by antisera against CI and 49k NI (Fig. 11). Due to a lack of evidence in the RRL system to support linkages of the 48k HC and 42k unknown products, the organization of this particular part of BYMV genome was not conclusive. Fortunately, in the WG system we detected a 110k product which could only be immunoprecipitated by antiserum to HC, indicating that the gene adjacent to HC must be an unknown gene instead of CI. If the 73k CI were adjacent to HC, then this 110k product should be immunoprecipitated by antisera to CI and HC. This observation confirmed that the proposed order (e.g., HC, unknown protein and CI) for the internal part of BYMV genome was

correct. However, based on Mr. estimation of this 110k product the Mr. of the unknown gene next to HC should be around 30k instead of 42k as proposed using data from the RRL system. The 115k product immunoprecipitated only by antiserum to CI was consistently found in both RRL and WG systems, which strongly suggests that this particular part of the genome should contain 42k of unknown protein sequences in addition to 73k CI. If it is true that the Mr. of the unknown gene next to HC is 30k, then two possible gene orders from 5' downstream to 3' for this part of the BYMV genome could be (1) 30k unknown gene, 73k CI and 12k unknown gene; or (2) 30k unknown gene, 12k unknown gene and 73k CI (Fig. 14). We prefer the first order based on the finding that a 140k product, immunoprecipitated by antisera to CI and 49k NI, was consistently detected in both the WG and RRL systems. This product might represent the polyprotein containing 73k CI, 12k unknown product and 49k NI. Although we have also found a 120k product in both systems, representing a polyprotein of 73k CI and 49k NI, the signal of the 140k product was consistently much stronger than this 120k product. Consequently, the 120k product might be a prematurely terminated polyprotein containing 73k CI, 12k unknown protein and part of the 49k NI. However, we have not ruled out the possibility of the second proposed gene order because immunological probes to the unknown products are still unavailable to show the linkages of the unknown gene to the CI or 49k NI. Nevertheless, strong evidence has been provided in this chapter that the 49k NI protein should be mapped next to the 54k NI protein and that there should be an unknown gene in between the HC and CI genes. Further experimentation would be

required to clarify if there is a small unknown gene in between the CI and 49k NI gene.

CHAPTER 4
INHIBITION OF PROTEOLYTICAL PROCESSING OF POLYPROTEINS TRANSLATED IN VITRO: EVIDENCE FOR THE 49K NUCLEAR INCLUSION PROTEIN OF BEAN YELLOW MOSAIC VIRUS AS A VIRUS-SPECIFIED PROTEASE

Introduction

The genome structure of potyviruses is similar to animal picornaviruses and plant comoviruses in that (1) a genome linked protein (VPg) is attached to the 5'-terminus of the single-stranded RNA and (2) the RNA has a 3'-terminal poly (A) tail (Hari et al., 1979; Hari, 1981; Goldbach and van Kammen, 1985). Regardless of the extreme differences in their biological properties, picornaviruses and comoviruses share not only common genome structures but also a common translation strategy (Goldbach and van Kammen, 1985). These viruses express their genome by first translating genome length polyproteins and then proteolytically processing them into primary cleavage products at the nascent chain stage. Most of the primary cleavage products undergo further cleavages to form mature structural and nonstructural proteins. Little was known about the mechanisms of primary cleavage until recently, when it was shown that a cellular enzyme was involved (Tian and Shih, 1986). Secondary cleavages that are mediated by virus specified proteases have been documented (Palmenberg et al., 1979; Franssen et al., 1984). The cleavage sites and the mechanism used for generating these viral enzymes have been

determined (Semler et al., 1981; Emini et al., 1982; Palmenberg and Ruckert, 1982).

In a study of in vitro translation of papaya ringspot virus RNA, a 330k product, representing probably the full length polyprotein, as well as the precursor and product relationship were found in the RRL without the incorporation of dithiothreitol (DTT) (Yeh and Gonsalves, 1985). This indicated polyprotein processing similar to that of picornaviruses and comoviruses, as a translation strategy of potyviruses. Similar results also have been established with several different potyviruses (Chang et al., 1985a; Xiong et al., 1985; E. Hiebert, unpublished data). Recently, direct evidence for polyprotein processing of potyviruses was provided by a sequencing study of TEV capsid protein gene (Allison et al., 1985). No stop codon was found in between the sequences for 54k NI protein and capsid protein of TEV. Consequently, it was shown the precursor, a polyprotein, for these two proteins was cleaved at a glutamine-glycine site to yield mature 54k NI protein and capsid protein. However, evidence for the existence of protease encoded by potyviral RNA has not yet been presented. In this chapter, we have shown antiserum to 49k NI protein of BYMV inhibited the proteolytical processing of some high Mr. proteins into low Mr. proteins in the RRL translation lysate. This result provides the first evidence that a potyviral protein is responsible for the protease activity of polyprotein processing.

Materials and Methods

Virus Source and Propagation

The PV-2 isolate of BYMV (Schroeder and Provvidenti, 1966) was used throughout the study. The virus was routinely propagated in 'Alaska' pea and maintained in a constant 26 C growth room.

Virus and RNA Purification

Purification of BYMV-PV-2 was performed as described by Nagel et al. (1983) except that Hepes buffer (20 mM, pH 8.2, containing 10 mM EDTA) was used to resuspend virus preparations throughout the purification procedures. The RNA of BYMV-PV-2 was prepared as described in Chapter 3.

Preparation of Viral Proteins and Antisera

The NI, CI and capsid proteins of BYMV-PV-2 were purified as described in Chapter 2. The preparation of antisera to these viral proteins has been described in Chapter 2.

Lysate Preparation and In Vitro Translation

Lysate preparation and in vitro translation were done as described in Chapter 3.

Immunoprecipitation Analysis of Translation Products

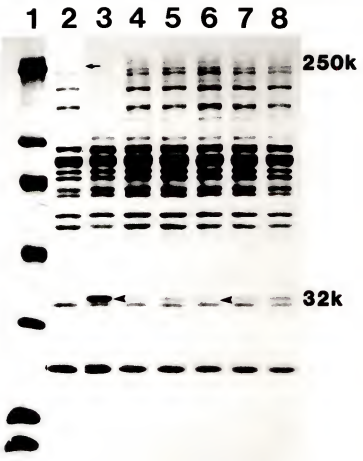
The procedures for immunoprecipitation analysis of in vitro translation product were the same as described in Chapter 3.

Results

Translation of BYMV-PV-2 RNA was first done in the RRL system without adding DTT. High Mr. products have been consistently obtained with this treatment (Chapter 3). After a 90-min incubation, an

aliquot of the lysate was taken as control and its reaction was stopped by adding 3 x vol of Laemmli dissociation solution (Chapter 3). The rest of the lysate was separated into six equal aliquots for inhibition experiments. Treatments included antisera to BYMV-PV-2 induced CP, CI, 54k NI and 49k NI, respectively, and preimmune serum (NS) as well as water as controls. Reaction mixtures contained 40 ul RRL lysate, 2 ul DTT and 5 ul of antiserum or NS. The reactions were incubated at 30 C for 2 hr and then stopped by adding 3 x vol of Laemmli dissociation solution. Translation products thus obtained were immunoprecipitated with antisera to CP, 49k NI and CI proteins, and analyzed in SDS-PAGE followed by fluorography. In the control lysate where water was substituted for antiserum, a product with Mr. greater than 200k as well as two other products with Mr. of 195k and 185k disappeared upon addition of DTT into the lysates (Fig. 33). A 32k product that was immunoprecipitated by antiserum to CP appeared after incubation with DTT (Fig. 33). This product was detected in the lysates treated with antisera against 54k NI, CP and CI but it was greatly reduced, although still detectable, in the lysate treated with 49k NI antiserum (Fig. 33). High Mr. products in the lysate treated with antiserum to 49k NI protein were much more intense than those in lysates treated with other antisera and NS. Lysate treated with NS still contained high Mr. products; however, the intensities of the bands were comparable to those in lysates treated with antisera to 54k NI, CI and CP (Fig. 33). This indicated NS might contain some unknown factors which could partially interfere with the cleavages of polyproteins. Similar results were obtained when lysates

Fig. 33. Immunoprecipitation analysis of capsid protein related products in BYMV-PV-2 stimulated rabbit reticulocyte lysates treated with different antisera to BYMV-PV-2 specified proteins. All lysate treatments were analyzed by immunoprecipitating with antiserum to BYMV-PV-2 capsid protein. The figure illustrates [S^{35}]methionine-labeled products separated in 7.5-15% SDS-PAGE and detected by fluorography. Translations were done without DTT incorporated. Lane 1, C^{14} standard proteins. Molecular weight markers in kilodaltons are from the bottom to the top: lysozyme (14.3k), lactoglobulin (18.4k), chymotrypsinogen (25.7k), ovalbumin (43k), bovine serum albumin (68k), phosphorylase b (97.4k) and myosin (200k). Lane 2, control lysate translation done without DTT; Lane 3, control lysate translation done without DTT, but DTT was added after translation and the mixture was incubated for 2 hr; the following lanes were lysates treated by adding DTT and antiserum to BYMV-PV-2 CP (lane 4), to BYMV-PV-2 CI protein (lane 5), to BYMV-PV-2 49k NI protein (lane 6), to BYMV-PV-2 54k NI protein (lane 7) and to normal serum (lane 8). The 250k product in lane 2 that disappeared in lane 3 when DTT was added is indicated by an arrow with a tail, while the 32k product that appeared after incubation with DTT is indicated by an arrow without a tail. Molecular weights of important products are indicated on the right side of the figure.


1 2 3 4 5 6 7 8
250k
32k

were analyzed by immunoprecipitating with antiserum to 49k NI protein (Fig. 34). When lysates were analyzed with antiserum to CI protein, more conclusive results were obtained (Fig. 35). In the control lysate, the disappearance of the three high Mr. products mentioned above was accompanied by the appearance of three products with Mr. of 115k, 73k and 70k when the DTT was added and followed by incubations. These three lower Mr. products were not detected in the lysate treated with 49k NI antiserum; however, they were apparently observed in lysates treated with other antisera and NS. The intensities of high Mr. products in lysate treated with 49k NI antiserum were also higher than those in lysates treated with other antisera and NS. These results provided evidence that BYMV-PV-2 encoded 49k NI protein or 49k NI-related protein was responsible for at least the proteolytical processing of 32k, 49k, 73k, 70k and 115 products.

Another experiment was performed to show further that the polyprotein processing could be inhibited by antiserum to 49k NI protein. Translations were done in the RRL system with and without DTT. Translation lysate with DTT, referrred to as WD-lysate hereafter, was divided into seven aliquots. One of the aliquots was taken as control and the reaction was stopped, 90 min after translation was performed, by adding Laemmli dissociation solution. The other six aliquots were treated with antisera to CP, CI, 54k NI and 49k NI protein, NS and water, respectively. The reaction mixture contained 10 ul lysate and 2.5 ul of antiserum, NS or water. After incubating the mixtures for 1 hr, 5 ul of mixtures were taken from

Fig. 34. Immunoprecipitation analysis of 49k NI related products in BYMV-PV-2 stimulated rabbit reticulocyte lysates treated with different antisera to BYMV-PV-2 specified proteins. All lysate treatments were analyzed by immunoprecipitating with BYMV-PV-2 49k NI protein antiserum. The figure illustrates [S^{35}]methionine-labeled products separated in 7.5-15% SDS-PAGE and detected by fluorography. Translations were done without DTT incorporated. Lane 1, C^{14} standard proteins. Molecular weight markers in kilodaltons are from the bottom to the top: lysozyme (14.3k); lactoglobulin (18.4k); chymotrypsinogen (25.7k); ovalbumin (43k), bovine serum albumin (68k), phosphorylase b (97.4k) and myosin (200k). Lane 2, control lysate translation done without DTT; Lane 3, control lysate translation done without DTT, but DTT was added after translation and the mixture was incubated for 2 hr; the following lanes were lysates treated by adding DTT and antiserum to BYMV-PV-2 CP (lane 4), to BYMV-PV-2 CI protein (lane 5), to BYMV-PV-2 49k NI protein (lane 6), to BYMV-PV-2 54k NI protein (lane 7) and to normal serum (lane 8). Products in lane 2 that disappeared in lane 3 when DTT was added are indicated by arrows with tail, while the 49k product that appeared after incubation with DTT is indicated by arrow without a tail. Molecular weights of important products are indicated on the right side of the figure.

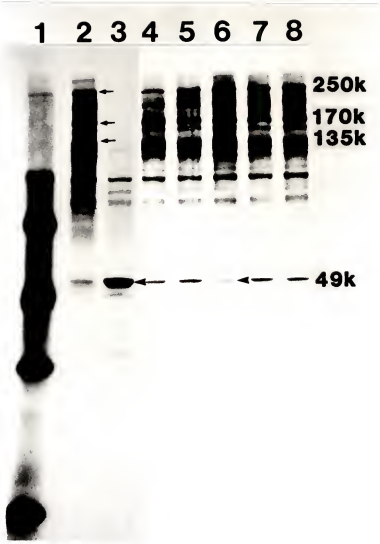
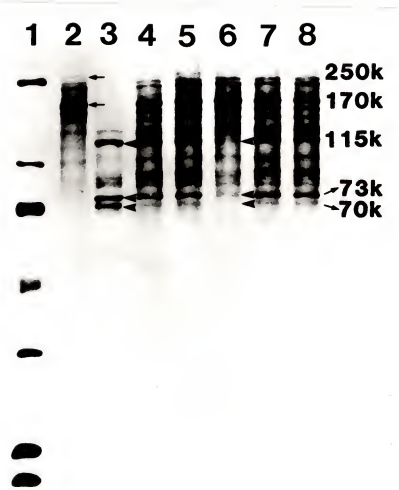

1 2 3 4 5 6 7 8
250k
170k
135k
49k

Fig. 35. Immunoprecipitation analysis of CI protein related products in BYMV-PV-2 stimulated rabbit reticulocyte lysates treated with different antisera to BYMV-PV-2 specified proteins. All lysate treatments were analyzed by immunoprecipitating with BYMV-PV-2 CI protein antiserum. The figure illustrates [S^{35}]methionine-labeled products separated in 7.5-15% SDS-PAGE and detected by fluorography. Translations were done without DTT incorporated. Lane 1, C^{14} standard proteins. Molecular weight markers in kilodaltons are from the bottom to the top: lysozyme (14.3k), lactoglobulin (18.4k), chymotrypsinogen (25.7k), ovalbumin (43k), bovine serum albumin (68k), phosphorylase b (97.4k) and myosin (200k). Lane 2, control lysate translation done without DTT; Lane 3, control lysate translation done without DTT, but DTT was added after translation and the mixture was incubated for 2 hr; the following lanes were lysates treated by adding DTT and antiserum to BYMV-PV-2 CP (lane 4), to BYMV-PV-2 CI protein (lane 5), to BYMV-PV-2 49k NI protein (lane 6), to BYMV-PV-2 54k NI protein (lane 7) and to normal serum (lane 8). The 250k and 170k products in lane 2 that disappeared in lane 3 when DTT was added are indicated by arrows with a tail, while the 115k, 73k and 70k products that appeared after incubation with DTT are indicated by arrows without a tail. Molecular weights of important products are indicated on the right side of the figure.


1 2 3 4 5 6 7 8
250k
170k
115k
73k
70k

each treatment and added to aliquots of 25 ul of translation lysates done without DTT, hereafter referred to as ND-lysate, followed by 2 hr incubation at 30 C before the reaction was stopped. The lysates were analyzed by immunoprecipitating with antisera to CI and CP. In the control ND-lysate where WD-lysate treated with water was added, high Mr. products disappeared with the appearance of low Mr. products mentioned above (Figs. 36 and 37). Similarly, intensities of high Mr. products were greatly reduced and several low Mr. products appeared in the ND-lysate when WD-lysate treated with NS was added. These two control treatments indicated that factors in WD-lysate could provoke processing of those high Mr. products, in the ND-lysate, into low Mr. products. This processing activity was inhibited by the addition of WD-lysate, treated with 49k NI antiserum, to the ND-lysate, but was not inhibited by incubating with the WD-lysates treated with other antisera (Figs. 36 and 37). This was shown by the reduced intensities of the 32k product, immunoprecipitated by antisera to CP, and the 70k, 73k and 115k products, immunoprecipitated by antiserum to CI. As before, the intensities of high Mr. products in lysate treated with 49k NI antiserum were higher than any other treatments, indicating that cleavages of high Mr. proteins into low Mr. proteins were inhibited.

Discussion

It has been shown already in Chapter 3 that by deletion of DTT from the RRL system, high Mr. in vitro translation products of BYMV-PV-2 can be retained. These high Mr. products are readily

Fig. 36. Immunoprecipitation analyses of capsid protein related products in ND-lysate incubated with WD-lysate treated with different antisera to BYMV-PV-2 specified proteins. The figure illustrates [S^{35}]methionine-labeled products separated in 7.5-15% SDS-PAGE and detected by fluorography. Translations were carried out with and without DTT added. Translation lysates done with DTT (WD-lysate) were divided into aliquots and treated with antisera to different BYMV-PV-2 specified proteins and normal serum. These treated WD-lysates were then separately added to translation lysate done without DTT (ND-lysate) followed by 2 hr incubation. All lysate treatments were analyzed by immunoprecipitating with antiserum to BYMV-PV-2 CP. Lane 1, C^{14} standard proteins. Molecular weight markers in kilodaltons are from the bottom to the top: lysozyme (14.3k), lactoglobulin (18.4k), chymotrypsinogen (25.7k), ovalbumin (43k), bovine serum albumin (68k), phosphorylase b (97.4k) and myosin (200k). Lane 2, control lysate translation done without DTT; Lane 3, control lysate translation done without DTT, but DTT was added after translation and the mixture was incubated for 2 hr; the following lanes were ND-lysates incubated with WD-lysate treated with antisera to BYMV-PV-2 CP (lane 4), to BYMV-PV-2 CI protein (lane 5), to BYMV-PV-2 49k NI protein (lane 6), to BYMV-PV-2 54k NI protein (lane 7), to normal serum (lane 8) and water (lane 9). The 250k product in lane 2 that disappeared in lane 3 when DTT was added is indicated by an arrow with a tail, while the 32k product that appeared after incubation with DTT is indicated by an arrow without a tail. Molecular weights of important products are indicated on the right side of the figure.

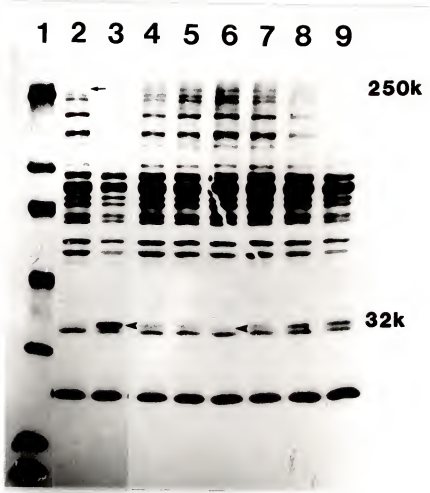
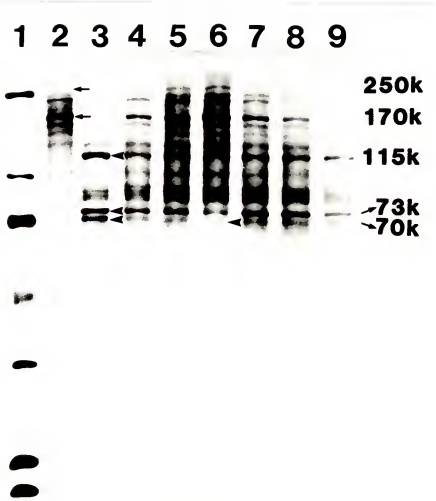

1 2 3 4 5 6 7 8 9
250k
32k

Fig. 37. Immunoprecipitation analyses of CI protein related products in ND-lysate incubated with WD-lysate treated with different antisera to BYMV-PV-2 specified proteins. The figure illustrates [S^{35}]methionine-labeled products separated in 7.5-15% SDS-PAGE and detected by fluorography. Translations were carried out with and without DTT. Translation lysates done with DTT (WD-lysate) were divided into aliquots and treated with antisera to different BYMV-PV-2 specified proteins and normal serum. These treated WD-lysates were then separately added to translation lysate done without DTT (ND-lysate) followed by 2 hr incubation. All lysate treatments were analyzed by immunoprecipitating with antiserum to BYMV-PV-2 CI protein. Lane 1, C^{14} standard proteins. Molecular weight markers in kilodaltons are from the bottom to the top: lysozyme (14.3k), lactoglobulin (18.4k), chymotrypsinogen (25.7k), ovalbumin (43k), bovine serum albumin (68k), phosphorylase b (97.4k) and myosin (200k). Lane 2, control lysate translation done without DTT; Lane 3, control lysate translation done without DTT, but DTT was added after translation and the mixture was incubated for 2 hr; the following lanes were ND-lysates incubated with WD-lysate treated with antisera to BYMV-PV-2 CP (lane 4), to BYMV-PV-2 CI protein (lane 5), to BYMV-PV-2 49k NI protein (lane 6), to BYMV-PV-2 54k NI protein (lane 7), to normal serum (lane 8) and water (lane 9). The 250k and 170k products in lane 2 that disappeared in lane 3 when DTT was added are indicated by arrows with a tail, while the 115k, 73k and 70k products that appeared after incubation with DTT are indicated by arrows without a tail. Molecular weights of important products are indicated on the right side of the figure.


1 2 3 4 5 6 7 8 9
250k
170k
115k
73k
70k

cleaved, accompanied with new appearance of certain lower Mr. products, once DTT is added. The disappearance of high Mr. products and/or appearance of certain lower Mr. products represent good indicators for determination of polyprotein processing activities. Using these indicators, we were able to show that antiserum to 49k NI protein of BYMV-PV-2 could inhibit polyprotein processing based on accumulation of more high Mr. indicator products than other treatments and disappearance of lower Mr. indicator products. Lysates treated with other antisera as well as preimmune serum were found to contain conspicuous amounts of low Mr. indicator products, indicating polyprotein processing had taken place. However, these treatments still contained, although less than those in lysate treated with 49k NI antiserum, detectable amounts of high Mr. indicator products. Accumulation of high Mr. indicator products in these treatments suggested that the sera might contain some unknown factors which could partially interfere with the cleavage of high Mr. products. This sugggestion was confirmed by the observation that less high Mr. products accumulated in the lysate treated with preimmune serum when we reduced the dosage of sera in the second experiment. This situation may also be improved by using purified immunoglobulins instead of crude sera.

In the second experiment, we showed that factors contained in the WD-lysate could provoke the processing of high Mr. indicator polyproteins into low Mr. indicator proteins, and the activities of these factors could only be inhibited by treating the WD-lysate with antiserum to 49k NI protein. Based on these results, we assumed that

these factors were 49k NI-related proteins. However, we still have not ruled out the possibility that the factors are just primary factors contained in RRL (Pelham, 1979; Tian and Shih, 1986), since DTT contained in WD-lysate may activate this factor to do primary cleavages. In further experiments, a WD-lysate without BYMV RNA will be included as a control and added to ND-lysate to see whether or not the high Mr. products can still be cleaved. This will allow us to differentiate if the processing is due to primary cleavage factors residing in the RRL or if it is due to the secondary cleavage factors encoded in the potyviral genome. We will also improve this experiment by using unlabeled methionine in WD-lysate to reduce the background problems caused by the [S^{35}]-labeled indicator products when [S^{35}]methionine was used in WD-lysate.

Another experiment will be performed by adding WD-lysates, which have been absorbed with different antisera, to ND-lysates. If the WD-lysate whose 49k-NI related protein has been removed by absorption can no longer provoke polyprotein processing in ND-lysates, then this would provide additional evidence for the protease activity of 49k NI protein.

In Chapter 2, we have shown that the plasmalemma is a possible site where potyviral protein synthesis occurs. We have also noticed that 49k NI protein or related antigens were detected on the plasmalemma at both an early stage of infection and at very late infection stages. This observation is in agreement with the protease function of 49k NI protein. It is reasonable that 49k NI protein as a

protease should stay at the plasmalemma to process the potyviral polyproteins.

CHAPTER 5
ANTIGENIC PROPERTIES OF NUCLEAR INCLUSION PROTEINS AS A BASIS FOR IDENTIFYING BEAN YELLOW MOSAIC VIRUS

Introduction

The potyviruses group is the largest and most economically important of the 29 recognized plant virus group (Hollings and Brunt, 1981). The viruses can be transmitted by mechanical inoculation, but in nature the majority of viruses in this group are transmitted nonpersistently by aphids (Matthews, 1982). Some of the members in this group have very wide host ranges but most of the members have only narrow host ranges (Teakle and Pares, 1977). Control of diseases caused by potyviruses is very difficult due to the efficient transmission of these viruses from plant to plant. The most reliable measures for controlling potyviral diseases are growing virus free materials and utilizing disease resistance (Hollings and Brunt, 1981). Developing these control methods relies on good indexing techniques which can detect and differentiate potyviral infections efficiently and accurately.

Traditionally, potyviruses are classified by host range, symptomatology and serological properties. Host range and symptomatology can vary depending on different environmental conditions and the use of different cultivars. The use of indicator plants is very time-consuming and requires a lot of working space.

Serological methods are among the most efficient methods for identification, although most viruses within this group are serologically related even though they are different in other properties (Brandes, 1964; Purcifull and Shepherd, 1964; Shepard et al., 1974).

The problem of distinguishing BYMV and CYVV represents an especially good example of the difficulties in differentiation of potyviruses in general. Both viruses occur world-wide and they have wide and similar host ranges (Bos, 1970; Hollings and Stone, 1974). Results of serological tests in attempts to differentiate these two viruses were particularly confusing because controversial results were usually obtained by different research groups. Some investigators considered them as separate viruses (Matthews, 1982; Lawson et al., 1985), but some considered them as strains or serotypes of the same virus (Jones and Diachun, 1977). All the serological tests used so far were based on differentiations of capsid proteins since purified virus particles were used mainly as immunogens. Limitation of using antisera to capsid protein of potyviruses for differentiation has long been recognized (Gibbs, 1969; Shepard et al., 1974). Besides capsid proteins, which represent only 10% of the genome capacity of potyviruses, potyviruses are capable of inducing several different kinds of nonstructural proteins in the infected tissue (Edwardson, 1974; Christie and Edwardson, 1977; Hollings and Brunt, 1981). Among the nonstructural proteins, cylindrical inclusions have been recognized as a taxonomical characteristic of potyviruses (Fenner, 1976; Hamilton et al., 1981; Matthews, 1982). Furthermore,

serological properties of cylindrical inclusions have been shown to be valuable as a criterion to differentiate potyviruses (Purcifull et al., 1973; Yeh and Gonsalves, 1984; Quiot-Douine et al., 1986). Since BYMV and CYVV are so difficult to distinguish based on serological properties of capsid proteins, it might be worthwhile to use nonstructural proteins as differentiation criteria.

In addition to cylindrical inclusions, it has been documented that some strains of BYMV and CYVV induce nuclear inclusions in situ (Christie and Edwardson, 1977; Nagel et al., 1983). Using NI proteins as criteria for differentiation of these two viruses should also be feasible. The NI induced by BYMV-PV-2 and CYVV-P have been successfully purified and the antisera to the NI proteins have been prepared (Chang et al., 1985). In this chapter, we will demonstrate the differentiation of BYMV from CYVV based on distinctions in serological properties of NI proteins of these two viruses. The BYMV and CYVV were also found distinguishable by the serological properties of cylindrical inclusion proteins.

Materials and Methods

Virus Cultures

An isolate (BYMV-PV-2) of BYMV (Schroeder and Provvidenti, 1966) and an isolate (CYVV-P) of CYVV (Pratt, 1969) were used in the purification of NI, CI and CP proteins. Both viruses were routinely maintained in cultivar 'Alaska' pea. Other isolates used included BYMV-O (Ko and Zettler, 1985), BYMV-F (Derks et al., 1980), BYMV-G (Nagel et al., 1983), BYMV-204-1 (Jones and Diachun, 1977) and

BYMV-Scott (Jones and Diachun, 1977). The BYMV-PV-2, CYVV-P, BYMV-F and BYMV-G were kindly provided by F. W. Zettler; BYMV-O was obtained from N. -J. Ko. The BYMV-204-1 and BYMV-Scott were obtained from M. McLaughlin and O. W. Barnett, respectively.

Purification of CI, NI and CP Proteins and Preparation of Antisera

Purification of CI, NI and CP proteins from BYMV-PV-2 and CYVV-P and production of antisera to these proteins were described in Chapter 2.

SDS-Immunodiffusion Tests and Indirect ELISA

The SDS-immunodiffusion tests were conducted according to the methods described by Purcifull and Batchelor (1977). Procedures of indirect ELISA were described in Chapter 2.

Results

Serological Relationship Between BYMV and CYVV NI Proteins

The NI induced by BYMV-PV-2 contained two distinct types of protein monomers with Mr. of 54k and 49k, respectively (Chapter 2). Likewise, NI induced by CYVV-P contained two distinct species of protein monomers but the Mr. of the large monomer was 60k and the small monomer was 49k (Chapter 2). Antisera were prepared against these NI proteins. These antisera reacted specifically with their own homologous antigens. No cross-reactivity to heterologous NI protein monomer, to CI protein, to capsid protein or to healthy plant extract was observed (Chapter 2).

When antiserum against 54k NI protein of BYMV-PV-2 was used in SDS-immunodiffusion tests, no antigenic differences between BYMV-PV-2 and the other two strains of BYMV tested (e.g., BYMV-204-1 and BYMV-Scott) were detected (Fig. 38). However, this antiserum consistently distinguished BYMV-PV-2 from CYVV-P because the precipitation line of BYMV-PV-2 spurred over that of CYVV-P (Fig. 38). These results were consistently obtained with every bleeding of this antiserum throughout a period of 1 year (Fig. 38). When six different strains of BYMV were tested against this antiserum, a continuously fused precipitation line was formed (Fig. 39), indicating that all BYMV strains tested were capable of inducing 54k NI protein related antigens in infected tissue and all these antigens were serologically indistinguishable by this test. Furthermore, the precipitation lines of three strains of BYMV (e.g., BYMV-PV-2, BYMV-Scott and BYMV-204-1) tested consistently spurred over the precipitation line formed by CYVV-P (Fig. 39).

In reciprocal tests, antiserum to CYVV-P 60k NI protein consistently detected differences between CYVV-P and three strains of BYMV (e.g., BYMV-PV-2, BYMV-204-1 and BYMV-Scott) with the homologous precipitation line spurring over the heterologous precipitation lines (Fig. 40). When the antiserum was tested against six different strains of BYMV, a continuously fused precipitation line also was observed (Fig. 40), confirming that large NI proteins in six different BYMV strains were serologically indistinguishable.

Antiserum to BYMV-PV-2 49k NI protein reacted with BYMV-Scott and BYMV-204-1 with very minor spur reactions between homologous and

Fig. 38. Serological relationship between BYMV-PV-2, BYMV-204-1, BYMV-Scott and CYVV-P as determined by SDS-immunodiffusion tests using different bleedings of antiserum to BYMV-PV-2 54k NI protein. The SDS-treated extracts of virus infected 'Alaska' pea were used as antigens to react with undiluted antiserum. Center wells were 1a, bleeding collected 1 month after final injection; 1b, bleeding collected 6 months after final injection; 1c, bleeding collected 1 year after final injection. Peripheral wells contained C, CYVV-P; P, BYMV-PV-2; S, BYMV-Scott; R, BYMV-204-1; H, SDS-treated healthy pea extract.

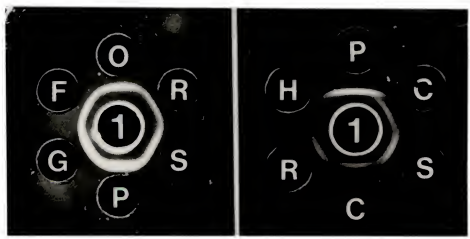


Fig. 39. Serological relationships between six different strains of BYMV and CYVV-P as determined by SDS-immunodiffusion tests using antiserum to BYMV-PV-2 54k NI protein. The SDS-treated extracts of virus infected 'Alaska' pea were used as antigens to react with undiluted antiserum. Wells contained O, BYMV-O; R, BYMV-204-1; S, BYMV-Scott; P, BYMV-PV-2; G, BYMV-G; F, BYMV-F; C, CYVV-P; H, SDS-treated healthy pea extract; 1, antiserum to BYMV-PV-2 54k NI protein.

Fig. 40. Serological relationships between six different strains of BYMV and CYVV-P as determined by SDS-immunodiffusion tests using antiserum to CYVV-P 60k NI protein. The SDS-treated extracts of virus infected 'Alaska' pea were used as antigens to react with undiluted antiserum. Wells contained O, BYMV-O; R, BYMV-204-1; S, BYMV-Scott; P, BYMV-PV-2; G, BYMV-G; F, BYMV-F; C, CYVV-P; H, SDS-treated healthy pea extract; 1, antiserum to CYVV-P 60k NI protein.

heterologous antigens in SDS-immunodiffusion tests (Fig. 41). These minor differences were consistently detected using different bleedings of this antiserum (Fig. 41). This antiserum, however, readily distinguished BYMV-PV-2 from CYVV-P by the obvious homologous precipitation line spurring over the heterologous precipitation line (Fig. 41). Reciprocally, antiserum to 49k NI protein of CYVV-P also consistently differentiated CYVV-P from different strains of BYMV with very conspicuous spur reactions (Fig. 41). When antigens of BYMV-PV-2, BYMV-Scott and BYMV-204-1 were set adjacent to each other, a continuously fused precipitation line was formed against antiserum to CYVV-P 49k NI protein (Fig. 41), indicating 49k NI protein related antigens of BYMV strains were antigenically closely related.

Immunoreactivities of NI proteins in extracts of three selected strains of BYMV (e.g., BYMV-PV-2, BYMV-204-1 and BYMV-Scott) and CYVV-P were also compared in indirect ELISA tests. Using antisera to 54k NI and 49k NI proteins of BYMV-PV-2, all three strains of BYMV gave similar higher ELISA readings throughout the dilutions of coating antigens, while CYVV-P always gave apparently lower readings (Fig. 42). In reciprocal tests, antiserum to 60k NI and 49k NI proteins of CYVV-P consistently reacted to BYMV strains with lower ELISA readings compared to the higher ELISA readings to CYVV-P throughout the series of coating antigen dilutions (Fig. 42).

Serological Relationship Between BYMV and CYVV CI Proteins

Antiserum to BYMV-PV-2 induced CI protein was nonreactive with extracts of CYVV-P in SDS-immunodiffusion tests (Fig. 43). These

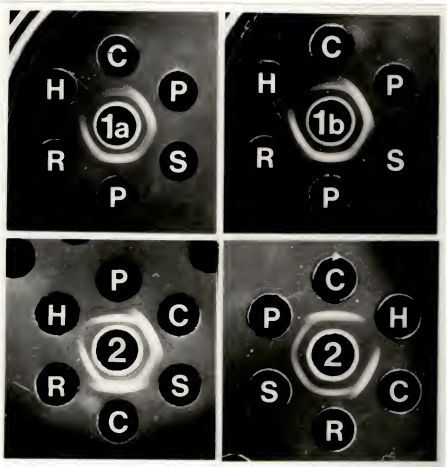


Fig. 41. Serological relationships between BYMV-PV-2, BYMV-204-1, BYMV-Scott and CYVV-P as determined by SDS-immunodiffusion tests using antisera to 49k NI proteins of BYMV-PV-2 and CYVV-P. The SDS-treated extracts of virus infected 'Alaska' pea were used as antigens to react with undiluted antiserum. Wells contained C, CYVV-P; R, BYMV-204-1; S, BYMV-Scott; P, BYMV-PV-2; H, SDS-treated healthy pea extract; 1a, bleeding of antiserum to BYMV-PV-2 49k NI protein collected 2 months after final injection; 1b, bleeding of antiserum to BYMV-PV-2 49k NI protein collected 6 months after final injection; 2, antiserum to 49k NI protein of CYVV-P.

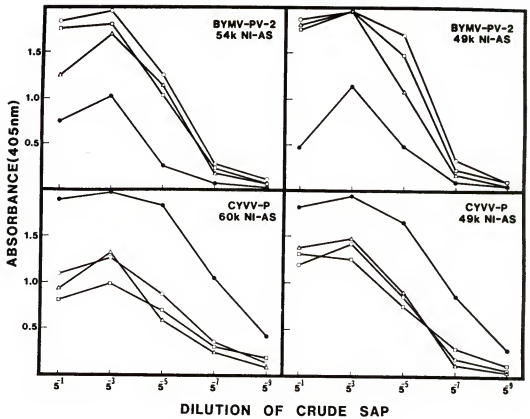


Fig. 42. Serological relationships between BYMV-PV-2, BYMV-204-1, BYMV-Scott and CYVV-P as determined by indirect ELISA using antisera to NI proteins of BYMV-PV-2 and CYVV-P. 'Alaska' pea tissue 15 days after inoculation with viruses tested were extracted with 5 vol of coating buffer. These extracts were further diluted by 5-fold series with coating buffer and used as coating antigens. Immunoglobulins to NI proteins of BYMV-PV-2 and CYVV-P were used at 1 ug/ml and goat anti-rabbit IgG alkaline phosphatase conjugate at 1/1,000 dilution. Readings were recorded 1 hr after adding substrate at 1 mg/ml. Antisera used for reactions are indicated on upper-right corner of each figure. ○—○ , readings of BYMV-PV-2 infected pea extract; □—□ , readings of BYMV-204-1 infected pea extract; △—△ , readings of BYMV-Scott infected pea extract; and ●—● , readings of CYVV-P infected pea extract. Readings seen on the figures represent values obtained after subtracting the readings of healthy control pea extract.

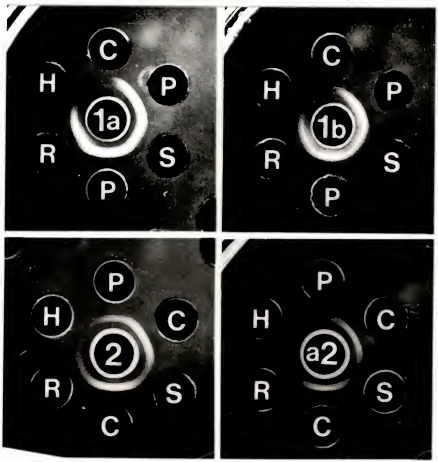


Fig. 43. Serological relationships between BYMV-PV-2, BYMV-204-1, BYMV-Scott and CYVV-P as determined by SDS-immunodiffusion tests using antisera to CI proteins of BYMV-PV-2 and CYVV-P. The SDS-treated extracts of virus infected 'Alaska' pea were used as antigens to react with undiluted antiserum. Wells contained C, CYVV-P; R, BYMV-204-1; S, BYMV-Scott; P, BYMV-PV-2; H, SDS-treated healthy pea extract; 1a, bleeding of antiserum to BYMV-PV-2 CI protein collected 4 months after final injection; 1b, bleeding of antiserum to BYMV-PV-2 CI protein collected 5 months after final injection; 2, antiserum to CYVV-P CI protein; a2, same antiserum as in 2 but intragel cross-absorption was performed with 1 mg/ml of PAGE-purified BYMV-PV-2 CI protein.

results were consistently obtained with different bleedings collected over a period of 8 months. Using these antisera, however, minor differences between BYMV-PV-2 and different strains of BYMV (e.g., BYMV-204-1 and BYMV-Scott) were detected with early bleedings up to 4 months after the final injection but no differences were observed with bleedings collected 5 months after the final injection (Fig. 43).

In reciprocal tests, antiserum to CI proteins of CYVV-P consistently reacted with both CYVV-P and three different strains of BYMV (e.g., BYMV-PV-2, BYMV-204-1 and BYMV-Scott) without perceptible spur reactions between homologous and heterologous antigens. However, the homologous reaction appeared to be much stronger than the heterologous reactions (Fig. 43). These heterologous reactions with different strains of BYMV disappeared while homologous reactions remained when intragel cross-absorptions were done with purified CI protein of BYMV-PV-2 (Fig. 43). This provided additional evidence that CI proteins CYVV-P and BYMV were serologically related to, but distinct from, each other, and that CI proteins of different BYMV strains were serologically very closely related to each other.

In indirect ELISA tests, despite types of coating antigen used (e.g., purified protein or crude extract), strains of BYMV always gave higher ELISA readings, while CYVV-P gave only lower readings, when tested with antiserum to BYMV-PV-2 CI proteins (Fig. 44). Reciprocally, different strains of BYMV responded similarly with antiserum against CYVV-P CI protein with lower ELISA readings, whereas this antiserum reacted homologously to CYVV-P with apparently higher ELISA readings (Fig. 44). These experiments again provided evidence

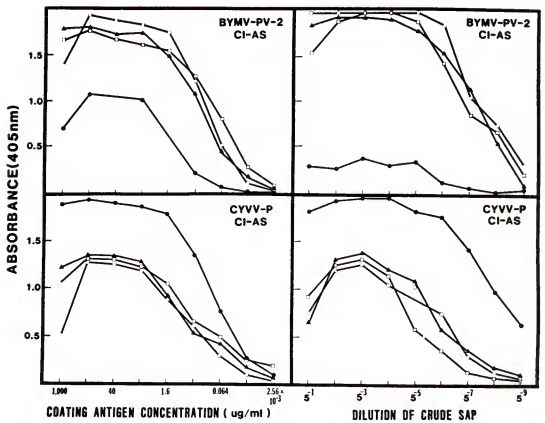


Fig. 44. Serological relationships between BYMV-PV-2, BYMV-204-1, BYMV-Scott and CYVV-P as determined by indirect ELISA using antisera to CI proteins of BYMV-PV-2 and CYVV-P. 'Alaska' pea tissue 15 days after inoculation with viruses tested were extracted with 5 vol of coating buffer. These extracts were further diluted by 5-fold series with coating buffer and used as coating antigens. When purified CI proteins of BYMV-PV-2 and CYVV-P were used as coating antigens, the proteins were diluted by 5-fold series with healthy pea crude sap extracted with coating buffer. Immunoglobulins to CI proteins of BYMV-PV-2 and CYVV-P were used at 1 ug/ml and goat anti-rabbit IgG alkaline phosphatase conjugate at 1/1,000 dilution. Readings were recorded 1 hr after adding substrate at 1 mg/ml. Antisera used for reactions are indicated on upper-right corner of each figure. o—o , readings of BYMV-PV-2 infected pea extract; □—□ , readings of BYMV-204-1 infected pea extract; ▲—▲ , readings of BYMV-Scott infected pea extract; ●—● , readings of CYVV-P infected pea extract. Readings seen on the figures represent values obtained after subtracting the readings of healthy control pea extract.

that wide antigenic disparity existed between the CI proteins of CYVV-P and BYMV, and that CI proteins of BYMV strains were very closely related to each other.

Serological Relationship Between BYMV and CYVV Capsid Proteins

Antiserum to BYMV-PV-2 CP reacted to BYMV-204-1, BYMV-Scott, BYMV-F, BYMV-G, BYMV-O, CYVV-P and homologously with BYMV-PV-2 with the homologous precipitation line always spurred over the heterologous precipitation line (Fig. 45). Differentiation of CYVV-P from BYMV was impossible in this case because this antiserum responded similarly when BYMV-PV-2 was compared with CYVV-P or with other strains of BYMV. The antiserum against CP of CYVV-P only reacted homologously and did not react with any of the BYMV strains tested (Fig. 45).

Serological Relationships Between BYMV and Other Potyviruses Based on Antigenic Properties of Large NI Protein

Serological relationship of the large NI protein of BYMV to other potyviruses was studied in SDS-immunodiffusion tests using the antiserum to 54k NI protein of BYMV-PV-2 (Fig. 46). Viruses used in this study included bean common mosaic virus (BCMV-S) (Lima et al., 1979), blackeye cowpea mosaic virus (BlCMV) (Lima et al., 1979), peanut mottle virus (PMoV) (obtained from J. Demski), cowpea aphid-borne mosaic virus (CAMV) (Lima et al., 1979) and watermelon mosaic virus 2 (WMV-2) (Purcifull and Hiebert, 1979). The results showed that extracts of BCMV-S, BlCMV and WMV-2 were not reactive with this antiserum, indicating these three viruses either did not induce or induced it in insufficient amounts to be detectable by

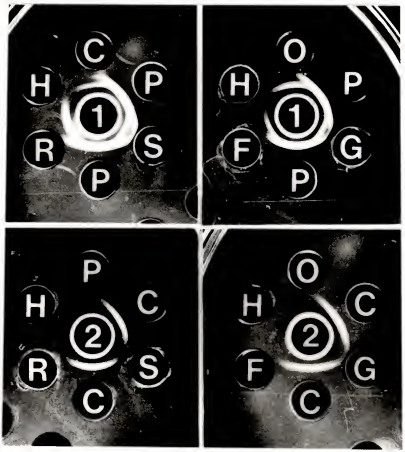


Fig. 45. Serological relationships between six different strains of BYMV and CYVV-P as determined by SDS-immunodiffusion tests using antiserum to capsid protein of BYMV-PV-2 and CYVV-P. The SDS-treated extracts of virus infected 'Alaska' pea were used as antigens to react with undiluted antiserum. Wells contained O, BYMV-O; R, BYMV-204-1; S, BYMV-Scott; P, BYMV-PV-2; G, BYMV-G; F, BYMV-F; C, CYVV-P; H, SDS-treated healthy pea extract; 1, antiserum to BYMV-PV-2 capsid protein; 2, antiserum to CYVV-P capsid protein.

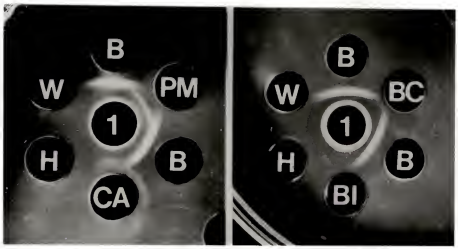


Fig. 46. Serological relationships between BYMV-PV-2 and other potyviruses as determined by SDS-immunodiffusion tests using antiserum to BYMV-PV-2 54k NI protein. The SDS-treated extracts of virus infected tissue were used as antigens to react with undiluted antiserum. Wells contained 1, undiluted antiserum to BYMV-PV-2 54k NI protein; B, BYMV-PV-2 infected pea extract; PM, peanut mottle virus infected pea extract; CA, cowpea aphid-borne mosaic virus infected blackeye cowpea extract; BI, blackeye cowpea mosaic virus infected blackeye cowpea extract; BC, bean common mosaic virus infected Bountiful bean extract; W, watermelon mosaic virus 2 infected pea extract; H, SDS-treated healthy blackeye cowpea tissue extract.

SDS-immunodiffusion tests. On the contrary, CAMV and PMoV were consistently detected inducing this type of protein in the infected tissue. Based on the spur formations between homologous and heterologous antigens, the large NI related proteins induced by these two viruses were serologically related to, but different from, the 54k NI protein of BYMV-PV-2.

Discussion

Bean yellow mosaic virus, as distinguished from most members of potyviruses, has a very wide host range. It causes diseases in various cultivated and wild legumes and also infects a number of nonlegumes including ornamental plants such as gladiolus and orchid (Bos, 1970). The world-wide distribution and economical importance of diseases caused by this virus have attracted the attention of plant pathologists from all over the world for years. It has long been realized that identification of BYMV is not a simple task for the following reasons: (1) Several closely related viruses have properties very similar to those of BYMV, thus making distinction of BYMV from these viruses very difficult. These viruses include CYVV (Hollings and Stone, 1974), pea necrosis virus (Beczner et al., 1976) and bean common mosaic virus (BCMV) (Bos, 1970). (2) Strains of BYMV have highly variable symptomatology, host range and serological properties, such as the pea mosaic strain (Schroeder and Provvidenti, 1966).

Since accurate identification of BYMV is problematic, a more definitive criterion for its identification would be useful. Along

with the capsid proteins, we have compared the serological relationships of BYMV and CYVV based on their capsid proteins and on three nonstructural proteins. These proteins represent about 70% of the genome capacity of potyviruses. Our results in comparison of capsid proteins between BYMV and CYVV and between BYMV strains were similar to those obtained by others (Jones and Diachun, 1977; Nagel et al., 1983; Barnett et al., 1985), in that all viruses tested including BYMV strains and CYVV-P were serologically related to, but distinct from, each other and the relationships between BYMV strains were not closer than that of BYMV to CYVV-P. The use of capsid protein antisera, therefore, would not be likely to permit the distinction of BYMV and CYVV isolates. Among the three nonstructural proteins (e.g., CI, large NI monomer and small NI monomer) tested, we found the antigenic properties of the large NI proteins were the most conserved among different strains of BYMV. Six different BYMV strains tested so far were all found to induce large NI protein in infected tissue and no differences in the antigenic properties of this protein were detected among these different strains. These strains included BYMV-PV-2 and BYMV-204-1, which was assigned as the third serogroup of BYMV (Jones and Diachun, 1977), but was regarded as a separate pea mosaic virus (PMV) by others (Reddick and Barnett, 1983) and BYMV-Scott, which was considered as the second serogroup of BYMV (Jones and Diachun, 1977) based on serological properties of capsid protein. In contrast, differences in serological properties between the large NI proteins of BYMV and CYVV were consistently detected. Furthermore, other viruses related to BYMV either did not induce detectable amounts

of NI protein in situ (e.g., BCMV) or they induced antigenically different large NI proteins (e.g., CAMV and PMoV). Consequently, using the antigenic properties of the large NI protein as a criterion we were able not only to distinguish BYMV from CYVV and from other viruses, but also to group BYMV strains. The significance of the conservation of this NI protein among different strains of the same virus is not understood so far; however, we speculate that this must have something to do with the conserved function of this protein since it has been claimed that the large NI protein of tobacco etch virus (TEV) possessed RNA polymerase activity (Hiebert and Dougherty, 1986).

Other nonstructural proteins tested (e.g., CI protein and small NI protein) are serologically variable among different strains of BYMV. However, the differences are so minor that spur reactions among BYMV-PV-2 and other strains of BYMV are not easily distinguished. Detection of these minor differences required selection of suitable bleedings of antisera and sometimes required application of intragel cross-absorption tests. On the contrary, obvious serological differences between BYMV-PV-2 and CYVV-P were consistently detected despite the bleedings of the antisera used and types of tests applied (e.g., SDS-immunodiffusion test or indirect ELISA). These results again provided evidence that using CI protein and small NI protein as criteria, strains of BYMV are serologically closer to each other than to CYVV-P.

Based on our results, pea mosaic virus (e.g., BYMV-PV-2 and BYMV-204-1), which was originally assigned as the third serogroup of BYMV (Jones and Diachun, 1977) or is considered as a separate virus by

others (Reddick and Barnett, 1983), is indistinguishable from BYMV by the serological properties of the large NI protein. It is also very closely related to other strains of BYMV in serological properties of CI protein and small NI protein. Therefore, it should be considered as a strain of BYMV instead of a separate virus. On the other hand, wide antigenic disparities were consistently detected in the three different nonstructural proteins as well as the capsid proteins between CYVV and BYMV. Therefore, CYVV should be recognized as a separate virus from BYMV instead of a strain or a serogroup of BYMV. This conclusion is in agreement with the classification of these viruses in the fourth report of the International Committee on Taxonomy of Viruses (Matthews, 1982).

CHAPTER 6
CONCLUSIONS

Nuclear inclusions (NI) induced by bean yellow mosaic virus (BYMV-PV-2) and clover yellow vein virus (CYVV-P) were successfully purified in this study. Both NI have similar staining properties and both are resistant to Triton X-100 treatment. Individually, the NI induced by CYVV-P are much smaller than those induced by BYMV-PV-2. From the top view BYMV-PV-2 NI are square-like while CYVV-P NI are diamond-shaped. The NI of BYMV-PV-2 and CYVV-P contain two distinct species of protein monomers. Similar results were obtained with tobacco etch virus (TEV) NI (Knuhtsen et al., 1974), and this is apparently a common phenomenon for potyviral NI. The molecular weights (Mr.) of the protein monomers of BYMV-PV-2 NI are 54k and 49k, while those for CYVV-P are 60k and 49k. The two NI protein monomers are serologically and chemically distinct from each other and from cylindrical inclusion and capsid proteins as determined by SDS-immunodiffusion test, Western blotting, immunofluorescence test, immunoprecipitation and peptide mapping.

Antisera to SDS-PAGE purified NI proteins of BYMV-PV-2 and CYVV-P reacted with NI in situ, indicating not only that these isolated NI proteins are the major constituent of the NI seen in situ, but also that SDS-treated NI proteins still preserve the antigenicities of the natural NI proteins.

The time course appearance study of the BYMV-PV-2 NI by immunofluorescence tests showed that the plasmalemma was possibly the site for potyviral proteins synthesis at the very early stage of infection. The size and number of NI are originally rather small but gradually increase with time. At the late stage of infection, the nuclei are always packed with NI and consequently they may rupture. Some of the Ni are apparently released from nuclei to the cytoplasm and become the so-called cytoplasmic crystals commonly found in BYMV infected tissue. These cytoplasmic crystals are antigenically and morphologically indistinguishable from the NI induced by BYMV. No cytoplasmic crystals similar to those of BYMV were found in CYVV-P infected tissue. However, a granular structure usually located adjacent to the nucleus in the CYVV-P infected cells were found equivalent to cytoplasmic crystals of BYMV, because they are also antigenically and morphologically indistinguishable to CYVV-P induced NI.

As with TEV NI, a 98k protein and a 100k protein are consistently associated with the NI preparations of BYMV-PV-2 and CYVV-P, respectively. This is also a common characteristic of potyviral NI isolated so far. These two proteins were shown containing sequences from both the large and small NI protein monomers by immunological analyses and by peptide mapping. Therefore, they are probably unprocessed polyproteins synthesized from BYMV-PV-2 and CYVV-P RNA. The significance of the existence of these unprocessed polyproteins in the infected tissue is still unknown to us, but detection of these polyproteins in vivo indicated that proteolytical processing of

polyproteins is part of the translation strategy of potyviruses. The evidence showing that these polyproteins contain sequences of both NI proteins also implicates the linkages of the two NI protein genes in the potyviral genome. This implication is in agreement with the result of genome mapping of BYMV-PV-2 RNA by immunoprecipitation analysis of the in vitro translation products in both the rabbit reticulocyte lysate (RRL) and the wheat germ (WG) system. In the RRL system, a product with MW of 135k immunoprecipitated by antisera to 49k NI, 54k NI and capsid protein was found, indicating that sequences encoding these three proteins are linked. Detection of products with Mr. of 170k, 150k, 140k and 120k, immunoprecipitated by antisera to CI and 49k NI, in both the RRL and the WG systems also indicates the linkage of CI and 49k NI protein genes in the BYMV-PV-2 genome. A 115k product immunoprecipitated only by antiserum to CI was consistently found in both the RRL and WG systems, implicating that there should be a sequence in BYMV-PV-2 genome that encodes the 73k CI and around 42k of unknown protein. At least 30k of this unknown protein is known to be linked with helper component protein coded in the sequences proximal to the 5'-terminus, because a 110k product was detected in the WG system that immunoprecipitated only by antiserum to HC. The rest of about 12k of unknown protein sequences may be located in between CI and 49k NI genes or in between the 30k unknown protein and CI genes. Although evidence obtained so far favors the former suggestion, additional experimentation seems required. The genome map for BYMV-PV-2 RNA from the 5'-terminus to the 3'-terminus is proposed as follows: 32k unknown protein, 48k HC, 30k unknown protein, 73k CI,

12k unknown protein, 49k NI, 54k NI and 32k CP. The precursor and product relationships and the proteolytic processing pathways are also presented.

With deletion of dithiothreitol (DTT) from the RRL system, our results were consistent with those of Yeh and Gonsalves (1985), in that a product with Mr. greater than 200k and a very high Mr. product, possibly corresponding to the full length 300k polyprotein, were found. These high Mr. products were readily converted to lower Mr. products once DTT was added, indicating that the proteolytical processing of polyproteins is the translation strategy of BYMV-PV-2 RNA. This suggestion is consistent with the detection of large amount of nonstructural proteins in the BYMV-PV-2 infected tissue.

Translation product profiles detected by immunoprecipitation analysis with antisera to BYMV-PV-2 specified proteins in the RRL and WG system were nearly identical. These findings are similar to those obtained with peanut mottle virus (Xiong et al., 1985) but differ from results obtained with other potyviruses (Hiebert and Dougherty, 1986). Nevertheless, a characteristic common to all potyviruses translated in the WG system was observed in our results in that the activity of the template proximal to the 5'-terminus is stronger in the WG system then in the RRL system. The reason for the difference in template activity of the same potyviral RNA in the RRL and the WG system is still not understood.

As mentioned, proteolytical processing of BYMV-PV-2 polyproteins in the RRL system can be provoked by treating the lysate with DTT. This is similar to the findings with plant comoviruses and animal

picornaviruses (Goldbach and van Kammen, 1985). In this study, we found the proteolytical processing activities provoked by adding DTT were inhibited by treating the lysate with antiserum to 49k NI but not by treating with antisera to other BYMV-PV-2 specified proteins. This provided the evidence showing that 49k NI protein associated with protease activities. Further experimentations are required to resolve some of the questions related to the mechanisms of the protease activities of 49k NI protein.

Numerous potyviruses encode proteins that react with TEV 54k NI protein antiserum (E. Hiebert, unpublished data). In this study, we demonstrated that the 54k NI protein of TEV, the 54k NI protein of BYMV-PV-2 and the 60k NI protein of CYVV-P, all representing the large monomers of NI, were serologically related. Furthermore, all three antisera to these large NI proteins immunoprecipitated an identical set of products from in vitro translation of a heterologous viral RNA (e.g., PeMV RNA), further confirming the serological conservativeness of the large NI proteins. This is apparently another common phenomenon associated with potyviral NI proteins.

Attempts to take advantage of the serological conservativeness of the large NI protein for identification of potyviruses in general were unsuccessful because it was found that not all potyviruses induced enough of this type of protein, in vivo, to permit its detection by ELISA. Therefore, it does not seem feasible to use this type of protein as a target for indexing potyviral infection. However, the large NI protein of BYMV-PV-2 was found antigenically very conserved among six different strains of BYMV tested, while the other two

nonstructural proteins (e.g., 49k NI and CI) and capsid proteins were antigenically variable among strains of BYMV. On the other hand, differences in serological properties of the BYMV 54k NI protein between BYMV and those of 11 other potyviruses were consistently detected. Consequently, using the antigenic properties of the large NI protein as a criterion we were able not only to distinguish other potyviruses from BYMV, but also to group BYMV strains. Among the four BYMV-PV-2 specified proteins tested, the capsid protein is the least reliable criterion for classification of BYMV, because of its high variability in antigenic properties among BYMV strains as well as between BYMV and other potyviruses. The 49k NI and CI proteins are also antigenically variable among strains of BYMV; however, the differences between BYMV strains are so minor that sometimes they are not easily perceived. On the contrary, obvious antigenic differences in these two proteins between BYMV and other potyviruses are always readily detected. In all, antigenic properties of nonstructural proteins are more conserved than those of capsid proteins among strains of BYMV. Consequently, the use of nonstructural proteins may be preferable to the use of capsid proteins as criteria for classification of BYMV isolates by serological tests.

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BIOGRAPHICAL SKETCH

Chin-An Chang was born in Taichung, Taiwan, The Republic of China on December 11, 1952. He graduated from primary school in September 1964, and from senior high school in September 1970.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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